

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
REQUEST FOR FILING NATIONAL PHASE OF
PCT APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495

To: Hon. Commissioner of Patents
 Washington, D.C. 20231

TRANSMITTAL LETTER TO THE UNITED STATES
 DESIGNATED/ELECTED OFFICE (DO/EO/US)

Atty Dkt: PM 0276611 /068800
M# /Client Ref.

From: Pillsbury Winthrop LLP, IP Group:

Date: January 16, 2001

This is a **REQUEST** for **FILING** a PCT/USA National Phase Application based on:

- | | | |
|--|---|---|
| 1. International Application

<u>PCT/GB99/02257</u>
<u>↑ country code</u> | 2. International Filing Date

<u>13 July 1999 (13.07.99)</u>
Day <u>MONTH</u> Year | 3. Earliest Priority Date Claimed

<u>13 July 1998 (13.07.98)</u>
Day <u>MONTH</u> Year
(use item 2 if no earlier priority) |
|--|---|---|
4. Measured from the earliest priority date in item 3, this PCT/USA National Phase Application Request is being filed within:

(a) ☐ 20 months from above item 3 date (b) ☒ 30 months from above item 3 date,

(c) Therefore, the due date (unextendable) is January 13, 2001

5. Title of Invention ARYLSULFONE LINKERS FOR MASS SPECTROMETRIC ANALYSIS

6. Inventor(s) Gunter Schmidt, Andrew Hugin Thompson, and Robert Alexander Walker Johnstone

Applicant herewith submits the following under 35 U.S.C. 371 to effect filing:

7. ☒ Please immediately start national examination procedures (35 U.S.C. 371 (f)).

8. ☒ **A copy of the International Application** as filed (35 U.S.C. 371(c)(2)) is transmitted herewith (file if in English but, if in foreign language, file only if not transmitted to PTO by the International Bureau) including:

- a. ☒ Request;
 b. ☒ Abstract;
 c. 50 pgs. Spec. and Claims;
 d. 16 sheet(s) Drawing which are ☐ informal ☒ formal of size ☒ A4 ☐ 11"

9. ☒ **A copy of the International Application has been transmitted by the International Bureau.**

10. **A translation of the International Application** into English (35 U.S.C. 371(c)(2))

- a. ☐ is transmitted herewith including: (1) ☐ Request; (2) ☐ Abstract;
 (3) _____ pgs. Spec. and Claims;
 (4) _____ sheet(s) Drawing which are:
☐ informal ☐ formal of size ☐ A4 ☐ 11"
- b. ☒ is not required, as the application was filed in English.
 c. ☐ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
 d. ☐ Translation verification attached (not required now).

11. ☒ **PLEASE AMEND** the specification before its first line by inserting as a separate paragraph:

- a. ☒ --This application is the national phase of international application PCT/GB99/02257
 filed July 13, 1999 which designated the U.S., and that international
 application ☒ was ☐ was not published under PCT Article 21(2) in English.--
 b. ☐ --This application also claims the benefit of U.S. Provisional Application No.
60/ _____, filed _____.--

12. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., **before 18th month** from first priority date above in item 3, are transmitted herewith (file only if in English) including:
13. ☒ PCT Article 19 claim amendments (if any) have been transmitted by the International Bureau
14. ☐ Translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., of **claim amendments** made before 18th month, is attached (**required by 20th month from the date in item 3 if box 4(a) above is X'd, or 30th month if box 4(b) is X'd, or else amendments will be considered canceled**).
15. **A declaration of the inventor** (35 U.S.C. 371(c)(4))
- a. ☐ is submitted herewith ☐ Original ☐ Facsimile/Copy
- b. ☒ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
16. **An International Search Report (ISR):**
- a. Was prepared by ☒ European Patent Office ☐ Japanese Patent Office ☐ Other
- b. ☒ has been transmitted by the international Bureau to PTO.
- c. ☒ copy herewith (7 pg(s).) ☐ plus Annex of family members (___ pg(s).).
17. **International Preliminary Examination Report (IPER):**
- a. ☒ has been transmitted (if this letter is filed after 28 months from date in item 3) in English by the International Bureau with Annexes (if any) in original language.
- b. ☒ copy herewith in English.
- c.1 ☐ IPER Annex(es) in original language ("Annexes" are amendments made to claims/spec/drawings during Examination) including attached amended:
- c.2 ☐ Specification/claim pages # ___ claims # ___
Dwg Sheets # ___
- d. ☐ Translation of Annex(es) to IPER (**required by 30th month due date, or else annexed amendments will be considered canceled**).
18. **Information Disclosure Statement** including:
- a. ☒ Attached Form PTO-1449 listing documents
- b. ☒ Attached copies of documents listed on Form PTO-1449
- c. ☒ A concise explanation of relevance of ISR references is given in the ISR.
19. ☐ **Assignment** document and Cover Sheet for recording are attached. Please mail the recorded assignment document back to the person whose signature, name and address appear at the end of this letter.
20. ☐ Copy of Power to IA agent.
21. ☐ **Drawings** (complete only if 8d or 10a(4) not completed): ___ sheet(s) per set: ☐ 1 set informal;
☐ Formal of size ☐ A4 ☐ 11"
22. Small Entity Status ☐ is **Not** claimed ☒ is claimed (**pre-filing confirmation required**)
- 22(a) (n/a) (No.) Small Entity Statement(s) enclosed (since 9/8/00 Small Entity Statements(s) not essential to make claim)
23. **Priority** is hereby claimed under 35 U.S.C. 119/365 based on the priority claim and the certified copy, both filed in the International Application during the international stage based on the filing in (country) Great Britain of:
- | | Application No. | Filing Date | | Application No. | Filing Date |
|-----|-----------------|--------------|-----|-----------------|-------------|
| (1) | 9815163.2 | 13 July 1998 | (2) | | |
| (3) | | | (4) | | |
| (5) | | | (6) | | |
- a. ☒ See Form PCT/IB/304 sent to US/DO with copy of priority documents. If copy has not been received, please proceed promptly to obtain same from the IB.
- b. ☐ Copy of Form PCT/IB/304 attached.
24. Attached:

25. Preliminary Amendment:

25.5 Per Item 17.c2, **cancel original** pages #____, claims #____, Drawing Sheets #26. **Calculation of the U.S. National Fee (35 U.S.C. 371 (c)(1)) and other fees is as follows:**Based on amended claim(s) per above item(s) ☐ 12, ☐ 14, ☐ 17, ☐ 25, ☐ 25.5 (hilit)

Total Effective Claims	39	minus 20 =	19	x \$18/\$9	=	\$171	966/967
Independent Claims	3	minus 3 =	0	x \$80/\$40	=	\$0	964/965
If any proper (ignore improper) Multiple Dependent claim is present,				add \$270/\$135		+135	968/969

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)): →→ **BASIC FEE REQUIRED, NOW** →→→→A. If country code letters in item 1 are **not** "US", "BR", "BB", "TT", "MX", "IL", "NZ", "IN" or "ZA"

See item 16 re:

- | | | |
|--|------------------|---------|
| 1. Search Report was <u>not prepared by EPO or JPO</u> ----- | add \$1000/\$500 | 960/961 |
| 2. Search Report was prepared by EPO or JPO ----- | add \$860/\$430 | 970/971 |
| | +430 | |

SKIP B, C, D AND E UNLESS country code letters in item 1 are "US", "BR", "BB", "TT", "MX", "IL", "NZ", "IN" or "ZA"

- ☒ B. If USPTO did not issue both International Search Report (ISR) and (if box 4(b) above is X'd) the International Examination Report (IPER), ----- add \$970/\$485 + 960/961
- (X) (only) (one) → ☐ C. If USPTO issued ISR but not IPER (or box 4(a) above is X'd), ----- add \$710/\$355 +0 958/959
- (of) (these) (4) → ☐ D. If USPTO issued IPER but IPER Sec. V boxes not all 3 YES, ----- add \$690/\$345 +0 956/957
- (boxes) → ☐ E. If international preliminary examination fee was paid to USPTO and Rules 492(a)(4) and 496(b) satisfied (IPER Sec. V all 3 boxes YES for all claims), ----- add \$100/\$50 +0 962/963

SUBTOTAL = \$736.00

28. If Assignment box 19 above is X'd, add Assignment Recording fee of ----\$40 +0 (581)

29. Attached is a check to cover the ----- **TOTAL FEES \$736.00**Our Deposit Account No. 03-3975
Our Order No.

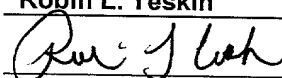
C#

M#

CHARGE STATEMENT: The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-18 and 492 (missing or insufficient fee only) now or hereafter relative to this application and the resulting Official document under Rule 20, or credit any overpayment, to our Account/Order Nos. shown above for which purpose a duplicate copy of this sheet is attached.

This CHARGE STATEMENT **does not authorize** charge of the **issue fee** until/unless an issue fee transmittal form is filedPillsbury Winthrop LLP
Intellectual Property Group1100 New York Avenue, NW
Ninth Floor
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Tel: (202) 861-3000
Atty/Sec: RLT/kmhBy Atty: Robin L. Teskin

Sig:

Reg. No. **35,030**Fax: **(202) 822-0944**
Tel: **(202) 861-3623****NOTE:** File in duplicate with 2 postcard receipts (PAT-103) & attachments.

ARYLSULFONE LINKERS FOR MASS SPECTROMETRIC ANALYSIS

This invention relates to methods of labelling analytes with markers that are cleavably detachable from their associated analytes and that are detectable by mass spectrometry. Specifically this invention refers to improved methods of detaching mass labels from their associated proteins, nucleic acids or other molecules of interest.

Cleavable mass labels have a number of advantages over other methods of detecting analytes with labels. In the analysis of DNA, commercially favoured systems are based on fluorescent labelling of DNA. Fluorescent labelling schemes permit the labelling of a relatively small number of molecules simultaneously, in which typically four labels are used simultaneously and possibly up to eight. However, the costs of the detection apparatus and the difficulties in analysing the resultant signals limit the number of labels that can be used simultaneously in a fluorescence detection scheme. An advantage of using mass labels is the possibility of making large numbers of labels, which can be discretely resolved in a mass spectrometer thereby allowing similar numbers of distinct molecular species to be labelled simultaneously. A feature of the use of cleavable mass labels disclosed is the need for linker groups that covalently link a mass marker to its corresponding nucleic acid whilst, at the same time being easy to detach from the analyte so as to permit detection of the mass marker.

PCT/GB98/00127 describes arrays of nucleic acid probes covalently attached to cleavable labels that are detectable by mass spectrometry which identify the sequence of a covalently linked nucleic acid probe. These mass labels have a number of advantages over other methods of analysing nucleic acids. The labelled probes of this application have the structure Nu-L-M where Nu is a nucleic acid covalently linked to L, a cleavable linker, covalently linked to M, a mass label. Preferred cleavable linkers in this application cleave within the ion source of the mass spectrometer, specifically within electrospray ion sources. Preferred mass labels are substituted poly-aryl ethers. This application discloses a variety of ionisation methods and analysis by quadrupole mass analysers, TOF analysers and magnetic sector instruments as specific methods of analysing mass labels by mass spectrometry.

PCT/GB94/01675 disclose ligands, and specifically nucleic acids, cleavably linked to mass tag molecules. Preferred cleavable linkers are photo-cleavable. This application discloses Matrix Assisted Laser Desorption Ionisation (MALDI) Time of Flight (TOF) mass spectrometry as a specific method of analysing mass labels by mass spectrometry.

PCT/US97/22639 discloses releasable non-volatile mass-label molecules. In preferred embodiments these labels comprise polymers, typically biopolymers which are cleavably attached to a reactive group or ligand, i.e. a probe. Preferred cleavable linkers appear to be chemically or enzymatically cleavable. This application discloses MALDI TOF mass spectrometry as a specific method of analysing mass labels by mass spectrometry.

PCT/US97/01070, PCT/US97/01046, PCT/US97/01304 disclose ligands, and specifically nucleic acids, cleavably linked to mass tag molecules. Preferred cleavable linkers appear to be chemically or photo-cleavable. These application discloses a variety of ionisation methods and analysis by quadrupole mass analysers, TOF analysers and magnetic sector instruments as specific methods of analysing mass labels by mass spectrometry.

There is still a need for improved linker groups having a number of properties:

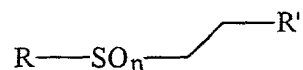
- A good linker must permit the mass marker to be separated from its nucleic acid with high efficiency prior to detection within a mass spectrometer.
- Cleavage of the label from its nucleic acid should preferably be performed in-line with a mass spectrometer, possibly after some in-line pre-fractionation step such as capillary electrophoresis. This in-line cleavage step preferably does not require a complex interface with the mass spectrometer to enable this step to occur. Ideally linkers should cleave at some predetermined point within existing instruments without any modification to the instrument beyond changes of normal operating parameters.
- Linkers should cleave under mild conditions without causing cleavage or other reactions in the associated nucleic acids hence reducing noise in the mass spectrum from nucleic acid fragmentation and ionisation.

- Linkers should all cleave under the same conditions to ensure all labels can be analysed simultaneously and quantitatively.
- Linkers should be compatible with a wide variety of mass labels.
- Linkers should preferably be attachable to a nucleic acid in multiple positions.
- Linkers should be stable under conditions within conventional automated oligonucleotide synthesisers.

It is an object of this invention to provide linkers that have the desired features disclosed above which are compatible with existing mass spectrometers particularly electrospray ionisation mass spectrometry. It is thus an object of the present invention to provide compounds that allow easy attachment and detachment of reporter groups, defined below, to and from analytes, particularly proteins and nucleic acids.

Accordingly, the present invention provides a method for characterising an analyte, which method comprises:

- (a) providing a compound in which the analyte is attached by a cleavable linker to a reporter group relatable to the analyte, the compound having the following formula:



wherein either R comprises the reporter group and R' comprises the analyte, or R comprises the analyte and R' comprises the reporter group, and wherein n is 1 or 2;

- (b) cleaving the reporter group from the analyte; and
- (c) identifying the reporter group, thereby characterising the analyte.

The cleavable linker used in this invention is not especially limited, provided that it is a group linking R to R' as set out in the above formula. The linker may comprise substituents on the carbon atoms, provided that the substituents do not prevent the linker from being cleavable. The linker may be cleaved, for example, in the ion source of a mass spectrometer by loss of a proton followed by intra-molecular rearrangements resulting in the breaking of a covalent bond in the linker. This is essentially a thermal process but can be promoted, for instance, by increasing the cone voltage within the ion source of an electrospray mass spectrometer. Thus the linker is preferably a thermally cleavable linker or a linker cleavable by electron impact.

The analyte molecule is not particularly limited, and may be any molecule of interest. Typically in the present invention, the analyte molecule comprises a biological molecule. Preferred biological molecules include a protein, a polypeptide, an amino acid, a nucleic acid (e.g. an RNA, a DNA, a plasmid, or an oligonucleotide), a nucleic acid base, and an organic molecule such as a pharmaceutical agent or a drug.

In the context of the present invention, the term amino acid is intended to broadly denote the twenty natural L-isomers of the alpha-amino acids, glycine, alanine, valine, leucine, isoleucine, serine, threonine, tyrosine, lysine, arginine, histidine, methionine, cysteine, phenylalanine, tryptophan, asparagine, glutamine, aspartic acid, glutamic acid and proline. It is also intended to denote all naturally modified forms of these amino acids. In addition the term amino acid is intended to denote the D-isomers of these amino acids and non-natural alpha amino acids that may be used as analogues whether in isolation or incorporated into synthetic or biosynthetic peptides.

The term polypeptide is intended to denote all polymers of amino acids, including dimers, short oligo-peptides, long polypeptides, whether synthetic or natural, and native proteins from biological samples, which may or may not be post-translationally modified. The term nucleotide is intended to denote both naturally occurring nucleotides and analogues, in particular the 2', 3' dideoxynucleotide analogues. The term polynucleotide is intended to

denote short oligonucleotides, long polynucleotides, whether synthetic or natural, and native nucleic acids from biological samples.

In the present invention, other analytes of interest may also include, for example, carbohydrates, lipids, drug molecules, natural products derived from living organisms and synthetic compounds from encoded chemical libraries.

In the context of the present invention, the term reporter group is intended to denote a moiety that is detectable by some conventional method of detection. The reporter group is preferably a mass marker, that is detectable by mass spectrometry. Appropriate reporters also include fluorophores, radiolabels, chemiluminescent labels, electron capture labels.

In the following description of the present invention, reference is made to 'intervening linker' or 'linker' groups which may be used to connect analytes or reporters to form the compounds of this invention. A variety of linkers is known in the art which may be introduced as further linkers between the thermally cleavable linkers of this invention and their covalently attached analyte or reporter molecules. Oligo- or poly-ethylene glycols or their derivatives are widely used as linkers (Maskos, U. & Southern, E.M. Nucleic Acids Research 20: 1679-1684, 1992) and may be used as the further linkers in the present invention. Succinic acid based linkages are also widely used although these are less preferred as they are generally base labile and are thus incompatible with the base mediated deprotection steps used in a number of oligonucleotide synthesisers.

Propargylic alcohol is a bifunctional linker that provides a linkage that is stable under the conditions of oligonucleotide synthesis and is a preferred further linker for use with this invention. Similarly 6-aminohexanol is a useful bifunctional reagent to link appropriately functionalised molecules and is also a preferred further linker.

A variety of cleavable linker groups is known in the art and may be used in conjunction with the compounds of this invention. Photocleavable linkers are well known in the art.

Ortho-nitrobenzyl groups are well known in the art as photocleavable linkers particularly 2-nitrobenzyl esters and 2-nitrobenzylamines, which cleave at the benzylamine bond. For a review on cleavable linkers see Lloyd-Williams *et al.*, Tetrahedron 49: 11065-11133, 1993, which covers a variety of photocleavable and chemically cleavable linkers.

When the analyte is a polynucleotide, the compounds of this invention can be formed by attaching a reporter group via a linker group to an polynucleotide at a number of locations in the polynucleotide. For conventional solid phase synthesisers the 5' hydroxyl of a terminal ribose or deoxyribose is the most readily accessible. Other favoured positions for modification are the 5' position in the pyrimidine heterocycle and the 7' position and 8' position in the purine heterocycle. These would all be appropriate positions to attach the compounds of this invention.

The 2' position on the ribose or deoxyribose heterocycle is also accessible for attachment of a linker group. This position can be modified to a considerable degree, including derivitisation with linkers to mass labels. The phosphate groups are also available for reaction with the compounds of this invention.

The compounds used in this invention are stable under conditions that occur in conventional protein or DNA analysis and under the conditions in automated peptide or oligonucleotide synthesisers. The compounds provided are compatible with a variety of mass spectrometric analytical techniques and ionisation methods such as matrix assisted laser desorption ionisation (MALDI), electrospray ionisation (ESI), thermospray ionisation or fast atom bombardment ionisation (FAB). Preferred mass spectrometry analytical techniques for use with the present invention also include pyrolysis and gas chromatography/mass spectrometry (GC/MS). Cleavage of the reporter from its protein or nucleic acid can be performed in-line with a mass spectrometer in some embodiments, possibly after a pre-fractionation step such as capillary electrophoresis (CE) or high performance liquid chromatography (HPLC). The in-line cleavage step does not require a complex interface with a mass spectrometer to enable this step to occur.

The invention will now be described in further detail by way of example only, with reference to the accompanying drawings, in which:

Figure 1 shows a generic linker for use in the present invention;

Figure 2 shows a model linker used in the present invention, FT 27;

Figure 3 shows a model linker used in the present invention, FT 28;

Figure 4 depicts a schematic of the synthesis of FT27 and FT 28;

Figure 5 depicts a schematic of the synthesis of CMM267B, a linker for attachment to the 5' hydroxyl group of a nucleotide or oligonucleotide;

Figure 6 depicts a schematic for the synthesis of FT48 and FT49;

Figure 7 depicts a schematic for attachment of a linker (FT49) to a nucleobase to form FT59;

Figure 8 shows the negative ion mass spectrum of FT28;

Figure 9 shows the probable mechanism by which F28 cleaves;

Figure 10 shows a schematic diagram of the synthesis of a series of mass labels that are cleavable in an electrospray ion source;

Figure 11 shows the electrospray mass spectrum of FT134 which is an ether mass marker that is attached to a linker that is cleavable in an electrospray ion source;

Figure 12 shows the electrospray mass spectrum of FT135 which is an ether mass marker that is attached to a linker that is cleavable in an electrospray ion source;

Figure 13 shows the electrospray mass spectrum of FT136 which is an ether mass marker that is attached to a linker that is cleavable in an electrospray ion source;

Figure 14 shows the electrospray mass spectrum of FT137 which is an ether mass marker that is attached to a linker that is cleavable in an electrospray ion source;

Figure 15 shows the electrospray mass spectrum of FT142 which is an ether mass marker that is attached to a linker that is cleavable in an electrospray ion source;

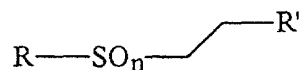
Figure 16 shows the electrospray mass spectrum of FT143 which is an ether mass marker that is attached to a linker that is cleavable in an electrospray ion source;

Figure 17 shows the electrospray mass spectrum of FT146 which is an ether mass marker that is attached to a linker that is cleavable in an electrospray ion source; and

Figure 18 shows the electrospray mass spectrum of FT147 which is an ether mass marker that is attached to a linker that is cleavable in an electrospray ion source.

The present methods will now be described in more detail. As mentioned above, the present invention provides a method for characterising an analyte, which method comprises:

- (a) providing a compound in which the analyte is attached by a thermally cleavable linker to a reporter group relatable to the analyte, the compound having the following formula:



wherein either R comprises the reporter group and R' comprises the analyte, or R comprises the analyte and R' comprises the reporter group, and wherein n is 1 or 2;

- (b) cleaving the reporter group from the analyte; and
- (c) identifying the reporter group, thereby characterising the analyte.

The analyte and/or reporter group may be attached directly to the linker (e.g. R (or R') consists of the analyte or the reporter group), or through a further group (e.g. R (or R') consists of the analyte or the reporter group plus a further group. The reporter and/or analyte may be attached directly to the linker or further group, or may be attached through a covalent linkage, as described below. Preferably R and/or R' should be stable during synthesis of the reporter group, during incorporation of the reporter group into the analyte, e.g. into an oligonucleotide in an automated synthesiser. Preferably R and/or R' should also be stable during the detection of the reporter group, e.g. under mass spectrometry. A wide variety of groups have these properties and might be incorporated into the linker. It may also be desirable to choose substituents which change the solubility of the linker.

As mentioned above, preferably, R and/or R' comprise a covalent linkage formed in attaching the analyte and/or reporter group to the cleavable linker. The covalent linkage is not particularly limited provided that the reporter group and/or the analyte can readily be attached to the cleavable linker using reactive functionalities attached to the linker and the reporter and/or analyte. Typically, both R and R' comprise a covalent linkage, although in some embodiments only R or only R' comprises a covalent linkage.

Table 1 below lists some reactive functionalities that may be reacted together to generate a covalent linkage between two entities. Any of the functionalities listed below could be used to form the compounds used in the present invention to permit the linker to be attached to an analyte (such as a nucleic acid or protein) and to an appropriate reporter group for detection

(e.g. by mass spectrometry). If desired, a reactive functionality can be used to introduce a further linking group with a further reactive functionality.

Table 1

Functionality 1	Functionality 2	Resultant Covalent Linkage
-NH ₂	-COOH	-CO-NH-
-NH ₂	-NCO	-NH-CO-NH-
-NH ₂	-NCS	-NH-CS-NH-
-NH ₂	-CHO	-CH ₂ -NH-
-NH ₂	-SO ₂ Cl	-SO ₂ -NH-
-NH ₂	-CH=CH-	-NH-CH ₂ -CH ₂ -
-OH	-OP(NCH(CH ₃) ₂) ₂	-OP(=O)(O)O-

It should be noted that some of the reactive functionalities above or their resultant covalent linkages might have to be protected prior to introduction into an oligonucleotide synthesiser. Preferably unprotected ether, ester, thioether and thioesters, amine and amide bonds are to be avoided as these are not stable in an oligonucleotide synthesiser. A wide variety of protective groups are known in the art to protect linkages from unwanted side reactions.

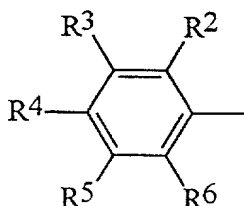
A short alkyl linkage would be appropriate to link the mass marker to the cleavable linker although a wide variety of linkers are available which can be used to link a mass marker to the tertiary amine group of the linker.

Thus, in preferred embodiments of the present invention, the covalent linkage attaching the cleavable linker to the reporter group and/or the analyte is independently selected from a -CO-NH- group, an -NH-CO-NH- group, an -NH-CS-NH- group, a -CH₂-NH- group, an -SO₂-NH- group, an -NH-CH₂-CH₂- group, or an -OP(=O)(O)O- group.

The R group in the compounds used in the present method is not particularly limited, provided that it comprises the reporter group or the analyte. The R group may thus comprise further groups, if desired. Typically R comprises, between the SO_n group and the reporter group or

analyte, a substituted or unsubstituted aromatic cyclic group, aliphatic cyclic group or heterocyclic group. Preferably R comprises a substituted or unsubstituted group selected from phenyl, pyridyl, pyranyl, naphthyl, anthracyl, pyrenyl, or fused ring derivatives or heteroaromatic analogues of the above. The substituents are not particularly limited and may comprise any organic group or a halogen (e.g. chlorine or bromine), preferably a hydrocarbon group, such as an alkyl group or a group comprising an alkene or alkyne functionality, a hydrocarbon group comprising a heteroatom, such as an N, O, P or S atom, or a cyclic group, such as an aromatic, aliphatic or heterocyclic group. The substituents may comprise a plurality of functional groups and may comprise straight chain or branched chain hydrocarbon groups.

When R comprises a phenyl group, the phenyl group is preferably a group having the following formula:

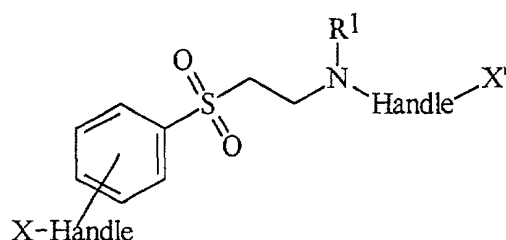


wherein one of R²-R⁶ comprises the reporter group or analyte, and the remaining R²-R⁶ groups are independently selected from a hydrogen, and a substituent as defined above, preferably a D, F, methyl, methoxy, hydroxy or amino group. It is particularly preferred that the R⁴ group comprises the reporter group or analyte.

The R' group in the compounds used in the present method is not particularly limited, provided that it comprises the reporter group or the analyte. The R' group may thus comprise further groups, if desired. Typically R' is a group selected from -S-, -SO-, -NR¹-, and -O- between the SO_n group and the reporter group or analyte. When the R' group comprises an -NR¹- group, preferably the R¹ group is an electron withdrawing group. More preferably, R¹ comprises a hydrogen atom, a halogen atom, or a substituent comprising a carbonyl group

and/or a halogen atom. Thus, R^1 may comprise a fluorine atom, a chlorine atom, a bromine atom, an iodine atom, a trifluoroacetyl group, a trifluoromethyl acetate group, a mesylate group or a tosylate group.

In a particularly preferred embodiment of the present method, the compound provided in step (a) of the method has the following formula:



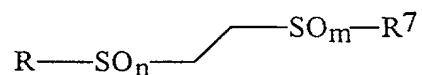
wherein R^1 is as defined above, X comprises the reporter group and X' comprises the analyte, or X comprises the analyte and X' comprises the reporter group, and each Handle is the same or different, being either a single bond directly attaching the X groups to the phenyl ring and the N atom respectively, or a covalent linkage as defined above.

In this embodiment of the present invention, the analyte is preferably attached to the linker through the phenyl group and the mass marker is preferably attached to the linker through the N atom. However, the analyte may also be attached to the linker through the N atom and the mass marker may also be attached to the linker through the phenyl group.

In this particular embodiment of the present invention, the analyte is preferably a nucleic acid or a nucleic acid base. Thus the analyte is preferably a DNA, an RNA an oligonucleotide or a plasmid. In this embodiment of the invention, the nucleic acid (or other analyte) is preferably attached to the linker through the phenyl group and the mass marker is preferably attached to the linker through the amine functionality.

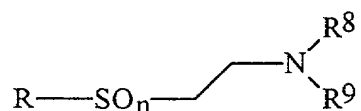
In the method of the present invention, the analyte under investigation is not particularly limited and may be any molecule of interest. Typically the analyte comprises a biological molecule. In preferred embodiments of the present invention, the biological molecule is selected from a protein, a polypeptide, an amino acid, a nucleic acid (e.g. an RNA, a DNA, a plasmid, or an oligonucleotide), a nucleic acid base, a pharmaceutical agent or drug, a carbohydrate, a lipid, a natural product and a synthetic compound from an encoded chemical library. When the analyte comprises a nucleotide, oligonucleotide or nucleic acid, the nucleotide, oligonucleotide or nucleic acid may be natural, or may be modified by modifying a base, sugar and/or backbone of the nucleotide, oligonucleotide or nucleic acid.

Polypeptides from natural tissue samples often have cysteine residues in them which may be cross linked with other cysteine residues in the same polypeptide resulting in a disulphide bridge or cysteine linkage. These disulphide bridges may be broken, exposing free thiols, prior to labelling of the polypeptides. This step may be effected by reduction with an appropriate reagent such as beta-mercaptoethanol or dithiothreitol. When the analyte is an amino acid or a peptide comprising a cysteine group, the compound provided in step (a) of the present method preferably has the following formula:



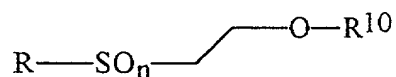
wherein R is as defined above and comprises a reporter group, m is 0 or 1 and the S atom attaching R⁷ to the linker is the sulphur atom of the cysteine group, R⁷ being the remainder of the amino acid or polypeptide. In the embodiment in which m=1, the sulphur of the cysteine has been oxidised. An appropriate reagent for this oxidation is, for example, hydrogen peroxide. This treatment renders the compound provided in step (a) more susceptible to thermal cleavage. This may be advantageous since certain types of analysis by mass spectrometry incorporate a step in which the compound is heated which would release the reporter group for analysis.

Alternatively, when the analyte is an amino acid or a peptide, the compound provided in step (a) may have the following formula:



wherein R is as defined above and comprises a reporter group, the N atom is the nitrogen atom of an epsilon amino group of a lysine group, or is the nitrogen atom of an N-terminal alpha amino group, R^8 is selected from H, O or an N-protective group, R^9 being the remainder of the amino acid or polypeptide. Where one of $-\text{R}^8$ or $-\text{R}^9$ is oxygen, the compound is particularly thermally labile and will undergo a Cope elimination on heating. The amine oxide may, for example be generated by reaction of the amine with hydrogen peroxide.

In a further alternative, when the analyte is an amino acid or a peptide comprising a serine, threonine and/or tyrosine group, the compound provided in step (a) may have the following formula:



wherein R is as defined above and comprises a reporter group, the O atom is the oxygen atom from a hydroxyl group of the serine, threonine or tyrosine group, R^{10} being the remainder of the amino acid or polypeptide.

The reporter group used in the present invention is not especially limited and may be any group, provided that it is readily detectable and can be related to an analyte to identify the

analyte. Typically, the reporter group is a mass marker, that is detectable by mass spectrometry. Other appropriate reporters include fluorophores, radiolabels, chemiluminescent labels, and electron capture labels.

In preferred embodiments of the present invention, mass markers disclosed in PCT/GB98/00127, PCT/GB98/03842, GB 9815166.5 and GB 9826159.7 can be employed. The content of these applications is incorporated by reference. PCT/GB98/00127 and PCT/GB98/03842 disclose poly-ether mass markers which are thermally stable, chemically inert and fragmentation resistant compounds, and which can be substituted with a variety of groups to alter properties such as solubility and charge. These mass markers are also preferred for use in the present invention and the content of this application is incorporated by reference. GB 9826159.7 discloses markers which comprise two components, which may be poly-ethers, which are analysed by selected reaction monitoring. These are particularly preferred mass markers for use in the present invention. GB 9815166.5 discloses mass markers that bind metal ions, which are also preferred markers for use with this invention. The content of this application is incorporated by reference. Reporter groups that can be detected by more than one detection means may also be desirable as with, for example, a fluorescent marker that incorporates a radioisotope in its linker and that is detectable by mass spectrometry and reporters of this kind are referred to as 'multi-mode reporter' groups. Preferred multi-mode reporter groups are detectable by mass spectrometry.

When the mass marker comprises an oligoether or a polyether, the oligoether or polyether may be a substituted or unsubstituted oligo- or poly-arylether. The oligoether or polyether preferably comprises one or more fluorine atom or methyl group substituents, or one or more ^2H or ^{13}C isotopic substituents.

It is further preferred that the mass marker comprises a metal ion-binding moiety. Typically, the metal ion-binding moiety comprises a porphyrin, a crown ether, hexahistidine, or a multidentate ligand. Preferably, the metal ion-binding moiety is a bidentate ligand or is EDTA. The metal ion-binding moiety may be bound to a monovalent, divalent or trivalent

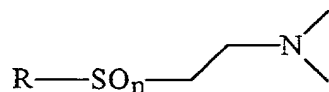
metal ion. The metal ion is not especially limited. Preferred metal ions include a transition metal ion, or a metal ion of group IA, IIA or IIIA of the periodic table. Particularly preferred metal ions are Ni^{2+} , Li^{+} , Na^{+} , K^{+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , or Al^{3+} . The presence of a metal ion on the mass marker increases the sensitivity of detection.

It should be noted that this invention is not limited to the mass markers disclosed above. A number of features are desirable in a molecule that is to be a good mass marker. In particular, it is preferred that a marker is:

- be easily detachable from the analyte, such as DNA,
- not interfere with enzymatic processes involved in molecular biology protocols,
- be fragmentation resistant in a mass spectrometer,
- form a single ion peak in the mass spectrum,
- permit very sensitive detection,
- be easily distinguishable from background contamination, such as DNA; it should be possible to determine that a mass peak is from a mass label and not from contaminants,
- be compatible with conventional automated oligonucleotide synthesisers,
- be easy to synthesise in a combinatorial manner to minimise number of chemical steps and the number of reagents necessary to generate large number of labels, and
- be compatible with existing mass spectrometry instrumentation without requiring physical modification of the instruments.

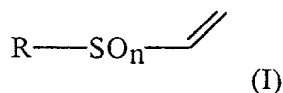
As mentioned above, the linkers of the present invention are preferably thermally cleavable linkers. Thus, the present method preferably further comprises a step of heating the linker to cleave off the reporter group. The method of heating is not especially limited. Preferably the linker is heated within the device for detecting the reporter group, e.g. within a mass spectrometer. The linkers used in the present invention are advantageous in that they are thermally cleavable under mild conditions. It is for this reason that the mass markers can be cleaved from a molecule of interest within the mass spectrometer itself.

However, cleavage is not limited to thermal cleavage and may also include chemical cleavage. For example, in those embodiments of the present method in which the compound provided in step (a) has the following formula:



wherein $n=1$ or 2 and R is as defined above, the following chemical reaction steps may be employed to induce cleavage:

- (i) reacting the above compound with an alkylating agent, to produce a quaternary ammonium derivative;
- (ii) reacting the resulting quaternary ammonium derivative with a base, to release a compound of formula:



wherein R and n are as defined above; and

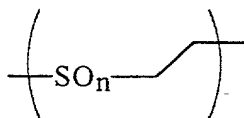
- (iii) detecting this resulting compound by mass spectrometry.

Appropriate methylating agents include dimethyl sulphate and methyl iodide. This step can be carried out in a solvent, such as methanol. An appropriate base includes diisopropylethylamine, which may be dissolved in a further organic solvent, such as dichloromethane. This reaction is a Hoffman elimination and is well known in the art.

As mentioned above, the thermal cleavage preferably takes place in (or in-line with) a mass spectrometer. Thermal cleavage may take place within the inlet to a gas chromatography instrument or within the inlet to a gas chromatography/mass spectrometer, a pyrolysis mass spectrometer, a thermospray mass spectrometer or an electrospray mass spectrometer. The thermal cleavage may also evaporate the sample into the gas phase. In some embodiments

thermal cleavage may be effected by laser, which may additionally desorb the reporter group for detection.

A second aspect of the present invention provides use of a linker group in the characterisation of an analyte, to attach a reporter group to the analyte, wherein the linker group is cleavable and has the following formula:



wherein n is 1 or 2.

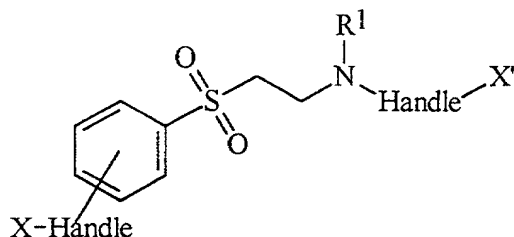
The linker is thus a cleavable linker, of the type described above in relation to the methods of the present invention. The analyte referred to in this aspect of the invention is not especially limited and may be any analyte as defined above. The reporter group referred to in this aspect of the invention is also not particularly limited and may be any reporter group as defined above.

The analyte and/or reporter group may be attached directly to the linker, or through a further group, such as the further groups described above. The reporter and/or analyte may be attached directly to the linker or further group, or may be attached through a covalent linkage, as described above.

In this aspect of the present invention, it is preferable that the linkers:

- cleave thermally under the conditions in an electrospray or thermospray ion source with a low cone voltage in an electrospray ion source, and/or
- are compatible with conventional oligonucleotide synthesis.

In a still further aspect, the present invention provides a compound having the following formula:



wherein R^1 is an electron withdrawing substituent, X comprises the reporter group and X' comprises the analyte, or X comprises the analyte and X' comprises the reporter group, and each Handle is the same or different, being either a single bond directly attaching the X groups to the phenyl ring and the N atom respectively, or a reactive group capable of attaching the X groups to the phenyl ring and the N atom respectively.

The compounds of this aspect of the invention are particularly preferred compounds of the type provided in step (a) of the method of the present invention. Thus, in the compounds of the present invention, $n=2$ and the group R defined above comprises a phenyl group substituted with a reporter group or analyte, attached to the phenyl group via a covalent linkage. Preferably the reporter group is attached to the phenyl group at a position *para* to the SO_2 - group. The group R^1 defined above comprises an $-NR^1$ - group attached via a covalent linkage to a reporter group or analyte.

In the compounds of the present invention the analyte is preferably attached to the linker through the phenyl group and the mass marker is preferably attached to the linker through the N atom. However, the analyte may also be attached to the linker through the N atom and the mass marker may also be attached to the linker through the phenyl group.

In this particular embodiment of the present invention, the analyte is not especially limited and is as defined above. Preferably the analyte is a nucleic acid or a nucleic acid base. Thus the analyte is preferably a DNA, an RNA an oligonucleotide or a plasmid. In this embodiment of the invention, the nucleic acid (or other analyte) is preferably attached to the linker through the phenyl group and the mass marker is preferably attached to the linker through the amine functionality.

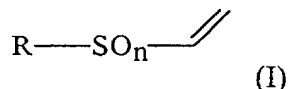
In the compounds of the present invention, R^1 is as defined above, each Handle is as defined above and the reporter group is as defined above.

Preferred compounds of the present invention are essentially mass labelled oligonucleotides, and are useful in genetic analysis such as sequencing, polymerase chain reaction (PCR) based assays, and gene expression profiling. Arrays of mass labels will allow arrays of oligonucleotides to be labelled. These may be used as sequencing primers or primers for PCR or as probes for hybridisation assays, where in each case the analysis of multiple samples may be multiplexed.

Formation of the compounds provided in step (a)

In a further aspect, the present invention provides a method for synthesising the compounds provided in step (a) of the present method.

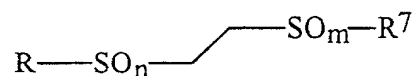
The method of this aspect of the invention comprises reacting a compound of the following formula:



wherein R and n are as defined above;

with one or more reporter groups or analytes.

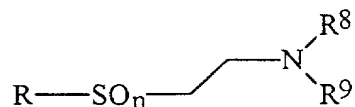
As mentioned above, when the analyte is an amino acid or a peptide comprising a cysteine group, the compound provided in step (a) of the present method preferably has the following formula:



wherein R is as defined above and comprises a reporter group, m is 0 or 1 and the S atom attaching R⁷ to the linker is the sulphur atom of the cysteine group, R⁷ being the remainder of the amino acid or polypeptide. These compounds may be produced according to this aspect of the present invention by reacting compounds of formula (I) with free thiols on one or more amino acids or polypeptides, which may need to be reduced.

In this embodiment of this aspect of the invention, it is preferred that the reaction takes place for a relatively short period of time, preferably a few minutes. Any free thiols in an amino acid or polypeptide will react readily with compound (I). Some polypeptides may need to be reduced to expose free thiols as discussed above.

When the analyte is an amino acid or a peptide, the compound provided in step (a) may have the following formula:



wherein R is as defined above and comprises a reporter group, the N atom is the nitrogen atom of an epsilon amino group of a lysine group, or is the nitrogen atom of an N-terminal alpha amino group, R⁸ is selected from H, O or an N-protective group, R⁹ being the

remainder of the amino acid or polypeptide. These compounds may be produced according to this aspect of the present invention by reacting compounds of formula (I) with free amines on one or more amino acids or polypeptides.

In this embodiment of this aspect of the present invention, production of the amine-attached derivatives may be accompanied by simultaneous production of sulphur-attached derivatives (discussed above). In this embodiment of this aspect of the invention, it is preferred that the reaction takes place for a relatively long period of time as compared with the reaction forming the sulphur-attached derivatives. Free amines are less reactive than free thiols to compounds of formula (I). If an amino acid or polypeptide is reacted with compounds of formula (I), free thiols will react most rapidly, while free amines will react more slowly. The reaction times will depend to some extent on the precise nature of the compounds of formula (I) and conditions used, which may be determined empirically. To selectively react amines rather than thiols, the thiols may need to be blocked. This may be effected by reaction of free thiols with 4-vinylpyridine or iodoacetic acid. Disulphides may be broken and capped with performic acid. These techniques are well known in the art.

When the analyte is an amino acid or a peptide comprising a serine, threonine and/or tyrosine group, the compound provided in step (a) may have the following formula:



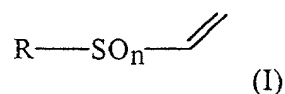
wherein R is as defined above and comprises a reporter group, the O atom is the oxygen atom from a hydroxyl group of the serine, threonine or tyrosine group, R¹⁰ being the remainder of the amino acid or polypeptide. These compounds may be produced according to this aspect of the present invention by reacting compounds of formula (I) with free hydroxyls on one or more amino acids or polypeptides.

In this embodiment of this aspect of the present invention, production of the oxygen-attached derivatives may be accompanied by simultaneous production of amine-attached and sulphur-attached derivatives (discussed above). In this embodiment of this aspect of the invention, it is preferred that the reaction takes place for an even longer period of time as compared with the reaction forming the sulphur-attached and amine attached derivatives. Free thiols and amines will react much faster with compounds of formula (I) than will free hydroxyls.

The method according to all of the embodiments of this aspect of the present invention may take place in an appropriate solvent. Appropriate solvents include acetonitrile or dimethylformamide. If a base is required, an appropriate base is triethylamine.

Applications of the mass markers used in the invention

The mass markers employed in the present invention are not especially limited. Markers produced after cleavage of the cleavable linker have a wide range of structure. As already mentioned above, one particular resulting marker structure is that of the following formula:



wherein R and n are as defined above.

These compounds are extremely useful reagents for analysis of amino acids, polypeptides, nucleotides, polynucleotides and other analytes of interest.

In particular, these compounds are useful in the analysis of amino acids and polypeptides by two dimensional (2-D) gel electrophoresis. 2-D gel electrophoresis is a technique for profiling protein expression, that is to say, cataloguing the identities and quantities of all the proteins expressed in a tissue (R.A. Van Bogelen., E.R. Olson, "Application of two-dimensional protein gels in biotechnology.", *Biotechnol Annu Rev*, 1:69-103, 1995). In this technique a

protein mixture, extracted from a biological sample, is separated on a narrow gel strip. This first separation usually separates proteins on the basis of their iso-electric point. The entire gel strip is then laid against one edge of a rectangular polyacrylamide gel. The separated proteins in the strip are then electrophoretically separated in the second gel on the basis of their size by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE). Once the separation is complete the proteins are visualised. This typically involves staining the gel with a reagent that can be detected visually or by fluorescence. Radiolabelling and autoradiography are also used. In other methods fluorescent dyes may be covalently linked to proteins in a sample prior to separation. Covalent addition of a dye can alter the mobility of a protein and so this is sometimes less preferred, particularly if comparisons are to be made with public databases of 2-Dimensional gel images. Having visualised the proteins in a gel it is usually necessary to identify the proteins in particular spots on the gel. This is typically done by cutting the spots out of the gel and extracting the proteins from the gel matrix. The extracted proteins can then be identified by a variety of techniques. Preferred techniques involve digestion of the protein, followed by microsequencing or more preferably the digested proteins are analysed by MALDI TOF to generate a peptide mass fingerprint. (Jungblut P, Thiede B. "Protein identification from 2-D gels by MALDI mass spectrometry." Mass Spectrom Rev. 16:145-162, 1997).

This methodology is very difficult to automate and it is also relatively insensitive in its simplest incarnations. At present 2-D analysis is a relatively slow 'batch' process. It is also not very reproducible and it is expensive to analyse a gel. Since most of the costs in a gel based analysis are in the handling of each gel it would be desirable to be able to multiplex a number of samples on a 2-D gel simultaneously. In principle, if it were possible to label the proteins in different samples with a different, independently detectable tag, then the proteins in each sample could be analysed simultaneously on the same gel. This would be especially valuable for studies where it is desirable to follow the behaviour of the same proteins in a particular organism at multiple time points, for example in monitoring how a bacteria responds to a drug over a predetermined time course. Similarly comparing biopsy material from multiple patients with the same disease with corresponding controls would be desirable to ensure that the same

protein from different samples would end up at the same spot on the gel. Running all the samples on the same gel would allow different samples to be compared without having to be concerned about the reproducibility of the separation of the gel. To achieve this requires a series of labels whose effect on the mobility of the proteins in different samples will be the same, so that a particular protein which is labelled with a different marker in each sample will still end up at the same position in the gel irrespective of its label. GB 9826159.7 discloses arrays of labels which will alter the mobility of their associated proteins to the same extent. The content of this application is incorporated by reference.

It is anticipated that compounds of the formula (I), where R comprises a mass marker of the type disclosed in GB 9826159.7, will be particularly effective for multiplexing 2-D PAGE analysis.

As mentioned above, the present invention also provides methods of forming compounds as defined in step (a) of the present methods by reacting a compound of the formula (I) with amino acids or polypeptides. Multiple samples can be labelled with different but related types of this compound that may be detected discretely by mass spectrometry. These labelled amino acids or polypeptides can then be separated by 2-D PAGE. In one method of analysing the resultant gel, the gel is electroblotted onto a target. The target is then raster scanned by laser so as to thermally cleave and desorb the reporter groups from their associated polypeptides for detection by mass spectrometry. In this method the whole gel or a region of the gel is completely imaged.

In an alternative embodiment, also employing compounds of formula (I), R may be a multi-mode reporter group, which comprises a mass marker of the type disclosed in GB 9826159.7 and a radio-isotope is incorporated into the marker. Polypeptides labelled with compounds of this form on a 2-D gel could be detected by autoradiography which will provide an image of the gel indicating where protein is present. Small samples from the gel can then be cored out from regions of interest for analysis by pyrolysis mass spectrometry. Pyrolysis is advantageous, in that purification of the sample could be reduced or avoided. In

preferred embodiments of the present invention, a radiolabel (e.g. ^{14}C) is incorporated in the cleavable linker, such that upon cleavage, the radiolabel remains in the analyte.

Examples

Synthesis of a model linker compound (FT28) that cleaves with high efficiency in ESI sources

A model linker, shown in Figure 2 was synthesised (see Figure 4). This compound is identified as FT27 hereafter.

Synthesis of FT27

A solution of phenyl vinyl sulphone (1.68 g, 10 mmol) and 6-aminohexanol (1.17 g, 10 mmol) in dry methanol (20 ml) was treated with triethyl amine (0.1 ml), refluxed for two hours, and stirred at room temperature overnight. The solvent was removed under reduced pressure. The oily residue was dissolved in ethyl acetate and the solvent was evaporated to give a crystalline residue. Recrystallisation from ethyl acetate/*n*-hexane yielded 2.67 g (94 %) FT 27.

Confirmation of Identity of FT27 Product

M.p. 60-61°C;

Calculated Atomic Composition: C 58.92, H 8.12, N 4.91; Measured Composition: C 58.91, H 8.14, N 4.89.

^1H NMR (CDCl_3) 1.30-1.55 (m, 10 H), 2.57 (t, $J = 7$ Hz, 2 H), 3.02 (t, $J = 7$ Hz, 2 H), 3.29 (t, $J = 7$ Hz, 2 H), 3.63 (t, $J = 7$ Hz, 2 H), 7.55-7.71 (m, 3 H), 7.90-7.96 (m, 2 H);

^{13}C NMR (CDCl_3) 25.44, 26.80, 29.69, 32.53, 43.03, 49.33, 56.02, 62.71, 128.01, 129.40, 133.84, 139.58.

Synthesis of FT28

A derivative of FT27 was synthesised (see Figure 4), identified as FT28 and shown in Figure 3, in which the amine group of FT27 was protected with a trifluoroacetate group.

A solution of FT27 (855 mg, 3 mmol) in dry dichloromethane (15 ml) and triethylamine (2.5 ml, 18 mmol) was treated at 0°C with trifluoroacetic anhydride (0.85 ml, 6 mmol) and stirred for 10 min at this temperature and then for 40 min at room temperature. The reaction mixture was poured into a mixture of phosphate buffer pH 7.0 (40 ml) and methanol (80 ml) and stirred for 15 min. The organic solvents were removed under reduced pressure and the remaining aqueous residue was treated with ethyl acetate. The organic phase was separated and washed with aqueous solutions of 5 % sodium bicarbonate, 10 % citric acid and water. The organic phase was dried with sodium sulphate and the solvents were removed under reduced pressure. The residue was purified by flash chromatography on silica gel with ethyl acetate/*n*-hexane (3:2) as eluent to afford 1.07 g (94 %) of FT28 as a waxy solid.

Confirmation of Identity of FT28 Product

¹H NMR (CDCl₃) 1.30-1.48 (m, 4 H), 1.50-1.68 (m, 4 H), 3.30-3.48 (m, 4 H), 3.65 (m, 2 H), 3.75 (t, *J* = 7 Hz, 2 H), 7.56-7.73 (m, 3H), 7.90-7.94 (m, 2H).

Synthesis of a linker for attachment to the 5'hydroxyl of a nucleotide or oligonucleotide

FT28 (0.306 g, 0.80 mmol) was co-evaporated three times with freshly distilled THF and then dissolved in THF (1.5 ml). The solution was stirred under argon and diisopropylethylamine (0.45 g, 0.6 ml, 3.5 mmol) was added. This was followed by the dropwise addition of 2-cyanoethyl-N,N-diisopropylchlorophosphoramidite (0.2 g, 0.2 ml, 0.85 mmol) and the reaction was stirred for one hour after which time Thin Layer Chromatography showed the reaction to be complete. The mixture was diluted (DCM), washed (KCl_(aq)), dried (Na₂SO₄) and then evaporated to dryness. The residue was dissolved in DCM (2 ml) then purified by precipitation into dry ice/acetone cooled hexane. The residue was dissolved in acetonitrile (1ml) and the solution was passed through a Gelman aerodisc (0.45 mm) then evaporated to dryness. The residual foam was co-evaporated several times with DCM before being dried in a vacuum desiccator over P₂O₅. This gave CMM267B (0.35 g, 75 %) as a white foam. $R_{fCMM267B} = 0.79$. $R_{fFT28} = 0.43$, DCM/EtOAc 1:1, U.V.

Synthesis of a linker for attachment to a nucleobase (FT49)

A model linker, shown in Figure 6 was synthesised via an intermediate identified as FT48. The model linker compound is identified as FT49 hereafter.

Synthesis of FT48

A solution of phenyl vinyl sulphone (1.68 g, 10 mmol), propargylamine (605 mg, 11 mmol) and triethylamine (0.1 ml) in methanol (20 ml) was refluxed for 2.5 hours. The solvent was removed under reduced pressure to afford 2.23 g (100 %) FT48 which was used without further purification.

Confirmation of Identity of FT48

¹H NMR (CDCl₃) 1.74 (br s, 1 H), 2.20 (t, *J* = 3 Hz, 1 H), 3.09 (t, *J* = 7 Hz, 2 H), 3.32 (t, *J* = 7 Hz, 2 H), 3.40 (d, *J* = 3 Hz, 2 H), 7.55-7.71 (m, 3 H), 7.92-7.96 (m, 2 H).

Synthesis of FT49

A solution of FT48 (2.0 g, 9 mmol) in dichloromethane (35 ml) was treated with triethylamine (2.5 ml), cooled to 0°C and subsequently treated with trifluoroacetic anhydride (2.3 g, 11 mmol). After stirring for 1 hour 40 min at this temperature the reaction was quenched with a saturated aqueous solution of ammonium chloride. The organic phase was separated, washed twice with water, dried with sodium sulphate, and evaporated to dryness under reduced pressure. The residue was purified by flash chromatography on silica gel with *n*-hexane/ethyl acetate (3:1) to furnish 2.77 g (96 %) FT49 as a colourless oil.

Confirmation of Identity of FT48

¹H NMR (CDCl₃) 2.30-2.40 (m, 1 H), 3.50 (m, 2 H), 3.93 (m, 2 H), 4.28 (m, 2 H), 7.54-7.76 (m, 3 H), 7.85-7.96 (m, 2 H).

Attachment of a linker to a nucleobase (FT59)

A solution of 5'-(4,4'-dimethoxytrityl)-5-iodouridine (656 mg, 1 mmol) in dry *N,N*-dimethylformamide (5 ml) was treated in sequence with a solution of FT49 (957 mg, 3 mmol) in dry *N,N*-dimethylformamide (5 ml), copper iodide (38 mg, 0.2 mmol), triethylamine (0.7 ml, 5 mmol), and palladium tetrakis(triphenylphosphine) (115 mg, 0.1 mmol). The reaction mixture was stirred for 6 hours at room temperature. The solvents were removed by co-evaporation with toluene under reduced pressure. The residue was dissolved in dichloromethane and washed with aqueous solutions of 5 % Na₂-EDTA, 10 % sodium thiosulphate, and water. The organic phase was dried with sodium sulphate and evaporated under reduced pressure. The residue was purified by flash chromatography on silica gel with dichloromethane/methanol (95:5) and subsequently with ethyl acetate/*n*-hexane/ethanol/triethylamine (65:30:5:1) to yield 650 mg (77 %) FT59 as a pale yellow foam.

Confirmation of Identity of FT59

Calculated Atomic Composition: C 60.91, H 4.75, N 4.96; Measured Composition: C 60.73, H 4.74, N 4.93.

¹H NMR (CDCl₃) 2.31 (m, 1 H), 2.51 (m, 1 H), 3.31-3.66 (m, 6 H), 3.79 (s, 6 H), 4.06-4.15 (m, 3 H), 4.52 (m, 1 H), 6.31 (m, 1 H), 6.84 (d, *J* = 10 Hz, 4 H), 7.20-7.67 (m, 14 H), 7.87 (m, 2 H), 8.10-8.18 (2 s, 1 H);

MS (FAB) *m/z* 848 [M+H]⁺.

Mass Spectrometry of model linkers FT27 and FT28

All data was acquired on a Platform-LC quadrupole instrument (Micromass Ltd, UK) with an electrospray ionisation source.

A solution of 5ng/ml of FT28 in a 50:50 mixture of water and acetonitrile was prepared. Ammonia was also present at 0.2 %. Figure 8 shows the negative ion mass spectrum of FT28. There is a relatively small peak at 380.1 corresponding to the molecular minus a single proton.

The region from 375-385 containing this peak has been magnified by 250x in Figure 8. There is a second much larger peak at 212.1 corresponding to the main cleavage product of FT28 that results from the ionisation of FT28. Figure 9 shows the probable mechanism by which the FT28 linker cleaves.

It should be noted that the trifluoroacetate group in FT28 protecting the amine linkage is unstable to strong base which is used in the final deprotection steps in an oligonucleotide synthesiser. This means that a different group must be found to replace this group which is stable to oligonucleotide synthesis to generate a linker that cleaves with high efficiency. Furthermore a reactive functionality must be substituted into the phenyl ring to provide the second handle required for this entity to be useful as a linker group.

Synthesis of eight model mass labels that are cleavable in an electrospray ion source

Eight ether compounds were synthesised from commercially available intermediates. These compounds were attached to linkers that cleave in an electrospray ion source. Two such linkers are used, one which contains a trifluoroacetate substitution protecting an amide group and one which contains a mesylate protective protecting the same amide group in the linker. Each of these linkers is attached to the same set of four ether mass labels to give a total of eight labels. Figure 10 shows a schematic of the syntheses performed. These mass labels were synthesised to demonstrate the principle of cleavage of labels in an electrospray ion source. The results show that the nature of the protective group is not essential to the functioning of the cleavable linker and that the nature of the mass marker does not interfere with the cleavage process. Thus this linker should be compatible with a wide variety of ether and poly-ether mass labels enabling the generation of large arrays of labels. The markers shown are model markers and are not attached to any analyte molecules. The core of the cleavable linker comprises a phenyl vinyl sulphone. The phenyl ring could be substituted with a bromine group, for example, to permit attachment to 1,7-octadiyne (Aldrich) which could then be used to react the marker group with 5'-(4,4'-Dimethoxy)trityl-5-iodouridine to generate nucleotide with a mass marker attached to a base. Similarly a brominated phenyl vinyl sulphone could be reacted with propargylic alcohol which may be O-protected (Aldrich) which would provide

a free hydroxyl to attach the mass labels to other positions within a nucleotide using standard methods known in the art. A variety of other groups might be introduced to enable these labels to be reacted with other analytes such as proteins, carbohydrates or other biomolecules.

Synthesis of FT116

A solution of *N*-benzyloxycarbonyl 6-aminocaproic acid (2.65 g, 10 mmol) in tetrahydrofuran (15 ml) was treated at 0°C with a 10 M solution of the borane dimethylsulphide complex in tetrahydrofuran (2.2 ml, 22 mmol) and stirred for 1 hour at 0°C and for 2 hours at room temperature. The reaction mixture was quenched by careful addition of methanol (2 ml). Subsequently the solvents were removed under reduced pressure and co-evaporated with methanol (3x20 ml) to give 2.416 g (96 %) of FT116. The crude product, which contains a non-polar impurity, was used without further purification in the next step.

An analytically pure sample was prepared by recrystallisation from ethyl acetate/*n*-hexane.

Confirmation of Identity of FT116 Product

M.p. 79-81°C

Calculated Atomic Composition C 66.90, H 8.42, N 5.57; Measured Composition C 67.17, H 8.68, N 5.67.

¹H NMR (CDCl₃): 1.35-1.64 (9 H, m), 3.19 (2 H, dt, *J* = 6.5 and 6.5 Hz), 3.62 (2 H, t, *J* = 6.5 Hz), 4.79 (1 H, br s), 5.10 (2 H, s), 7.26-7.37 (5 H, m).

¹³C NMR (CDCl₃): 25.31, 26.37, 29.96, 32.57, 40.95, 62.72, 66.64, 128.14, 128.57, 136.76, 156.57.

Mass spectrometry of the compound using Electron Impact Ionisation to ionise the compound gave peaks in the mass spectrum with the following mass to charge ratios: 251 (M⁺, <1 %), 160, 144, 130, 108, 91 (100 %).

Synthesis of FT117/2

A solution of FT116 (2.3 g, 9.2 mmol) in dichloromethane (45 ml) and dry triethylamine (5 ml) was treated at 0-5°C with methanesulphonyl chloride (1.375 g, 12 mmol). The reaction mixture was allowed to warm up to room temperature within 15 minutes and stirred for 4 hours at this temperature, diluted with dichloromethane (50 ml) and washed with a 5 % aqueous solution of sodium bicarbonate and water (2x). The organic phase was dried with sodium sulphate and the solvent was removed under reduced pressure. The residue was purified by flash chromatography on silica gel (100 g) using *n*-hexane/ethyl acetate (1:1) as eluent to furnish 2.413 g (80 %) of FT117/2.

Confirmation of Identity of FT117/2 Product

M.p. 24-27°C

Calculated Atomic Composition: C 54.69, H 7.04, N 4.25; Measured Composition: C 54.88, H 7.07, N 4.21.

¹H NMR (CDCl₃): 1.32-1.60 (6 H, m), 1.76 (2 H, m), 2.99 (3 H, s), 3.18 (2 H, m), 4.21 (2 H, t, *J* = 6.5 Hz), 4.76 (1 H, br s), 5.10 (2 H, s), 7.26-7.35 (5 H, m).

¹³C NMR (CDCl₃): 25.09, 26.08, 29.03, 29.82, 37.42, 40.89, 66.66, 69.84, 128.16, 128.58, 136.75, 156.51.

Mass spectrometry of the compound using Electron Impact Ionisation to ionise the compound gave ions with the following mass to charge ratios: 329 (M⁺, 3 %), 222, 194, 126, 108, 91 (100 %).

Synthesis of FT120, FT121, FT122, FT123 - General Procedure

A suspension of a 60 % oily suspension of sodium hydride (120 mg, 3 mmol) in *N,N*-dimethylformamide (3 ml) was treated with a solution of the corresponding phenol (3 mmol) in *N,N*-dimethylformamide (2 ml). After finishing of the hydrogen evolution a solution of FT117/2 (493 mg, 1.5 mmol) *N,N*-dimethylformamide (2 ml) was added. The reaction mixture was stirred at room temperature for 18 hours and diluted with diethyl ether/*n*-hexane (1:1, 25 ml). The solution was washed with water (2 x), 1 M aqueous

potassium hydroxide (3x10 ml) and water (2x). The organic phase was dried with sodium sulphate and evaporated to dryness. Flash chromatography on silica gel (20 g) using *n*-hexane/ethyl acetate (3:1) as eluent to afford the corresponding phenolether.

Confirmation of Identity of FT120 Product

FT120: yield 75 %; M.p. 79-81°C (diethyl ether/*n*-hexane)

Calculated Atomic Composition C 74.44, H 6.97, N 3.34; Measured Composition C 74.51, H 6.98, N 3.32.

^1H NMR (CDCl_3): 1.34-1.59 (6 H, m), 1.80 (2 H, t, $J = 6.5$ Hz), 3.21 (2 H, dt, $J = 6.5$ and 6.5 Hz), 3.93 (2 H, t, $J = 6.5$ Hz), 4.73 (1 H, br s), 5.10 (2 H, s), 6.81-7.06 (7 H, m), 7.17-7.37 (7 H, m).

^{13}C NMR (CDCl_3): 25.76, 26.48, 29.22, 29.54, 41.05, 66.66, 68.33, 115.59, 117.68, 120.83, 122.46, 128.15, 128.58, 129.65, 136.77, 150.18, 155.50, 156.49, 158.65.

Mass spectrometry of the compound using Electron Impact Ionisation to ionise the compound gave ions with the following mass to charge ratios: 419 (M^+ , 5 %), 311, 186 (100 %), 91, 77.

Confirmation of Identity of FT121 Product

FT121: yield 79 %, colourless oil

Calculated Atomic Composition C 69.54, H 7.00, N 4.06; Measured Composition C 69.34, H 7.04, N 3.99.

^1H NMR (CDCl_3): 1.37-1.5 (6 H, m), 1.76 (2 H, t, $J = 6.5$ Hz), 3.20 (2 H, dt, $J = 6.5$ and 6.5 Hz), 3.89 (2 H, t, $J = 6.5$ Hz), 4.73 (1 H, br s), 5.10 (2 H, s), 6.78-6.98 (4 H, m), 7.26-7.37 (5 H, m).

^{13}C NMR (CDCl_3): 25.72, 26.46, 29.17, 29.94, 41.03, 66.65, 68.49, 115.77, 115.62, 115.93, 128.15, 128.58, 129.65, 136.77, 155.29, 156.49, 158.85.

Mass spectrometry of the compound using Electron Impact Ionisation to ionise the compound gave ions with the following mass to charge ratios: 345 (M^+ , 5 %), 234, 202, 112, 91 (100 %).

Confirmation of Identity of FT122 Product

FT122: yield 72 %; M.p. 49-51°C (diethyl ether/*n*-hexane)

Calculated Atomic Composition C 74.33, H 8.22, N 3.94; Measured Composition C 74.61, H 8.33, N 3.88.

^1H NMR (CDCl_3): 1.25-1.57 (6 H, m), 1.75 (2 H, t, $J = 6.5$ Hz), 2.18 (3 H, s), 2.22 (3 H, s), 3.19 (2 H, dt, $J = 6.5$ and 6.5 Hz), 3.90 (2 H, t, $J = 6.5$ Hz), 4.73 (1 H, br s), 5.10 (2 H, s), 6.61-6.70 (2 H, m), 7.01 (1 H, d, $J = 8.2$ Hz), 7.25-7.36 (5 H, m).

^{13}C NMR (CDCl_3): 18.72, 19.77, 25.78, 26.49, 29.25, 29.95, 41.06, 66.63, 67.81, 111.52, 116.27, 128.13, 128.58, 130.33, 136.77, 137.92, 156.40, 157.33.

Mass spectrometry of the compound using Electron Impact Ionisation to ionise the compound gave ions with the following mass to charge ratios: 355 (M^+ , 15 %), 264, 246, 212, 122, 121, 107, 91 (100 %).

Confirmation of Identity of FT123 Product

FT123: yield 80 %; M.p. 42-44°C (diethyl ether/*n*-hexane)

Calculated Atomic Composition: C 76.36, H 7.21, N 3.71; Measured Composition: C 76.48, H 7.23, N 3.69.

^1H NMR (CDCl_3): 1.30-1.63 (6 H, m), 1.93 (2 H, t, $J = 6.5$ Hz), 3.20 (2 H, dt, $J = 6.5$ and 6.5 Hz), 4.11 (2 H, t, $J = 6.5$ Hz), 4.74 (1 H, br s), 5.10 (2 H, s), 6.78 (1 H, d, $J = 7.3$ Hz), 7.24-7.50 (9 H, m), 7.72 (1 H, m), 8.26 (1 H, m).

^{13}C NMR (CDCl_3): 26.01, 26.55, 29.23, 30.01, 41.09, 66.65, 67.99, 104.67, 120.11, 122.11, 125.15, 125.87, 125.96, 126.39, 127.51, 128.15, 128.58, 134.63, 136.97, 137.92, 154.94, 156.52.

Mass spectrometry of the compound using Electron Impact Ionisation to ionise the compound gave ions with the following mass to charge ratios: 377 (M^+ , 15 %), 269, 234, 165, 144, 127, 115, 91 (100 %).

Synthesis of FT126, FT128, FT130, FT132 - General Procedure

A solution of the corresponding *N*-benzyloxycarbonyl-protected phenolether (1 mmol) in methanol (20 ml) was treated with ammonium formate (250 mg, 4 mmol) and 10 % Palladium on charcoal (80 mg) and stirred for 15-60 minutes at room temperature. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated to dryness under reduced pressure. The residue was dissolved in water/ethyl acetate and 1 M potassium hydroxide was added. The organic phase was washed with 1 M potassium hydroxide, water (2 x) and dried with sodium sulphate. The solvents were removed under reduced pressure. The corresponding crude amines were used without further purification in the next step.

Synthesis of FT127, FT129, FT131, FT133 - General Procedure

A solution of the corresponding amine FT126, FT128, FT130 or FT132 (1 mmol) in methanol (10 ml) was treated with phenyl vinyl sulphone (168 mg, 1 mmol) and triethylamine (0.05 ml) and refluxed for 2 hours. The solvents were removed under reduced pressure and the residue purified by flash chromatography on silica gel (20 g) with ethyl acetate/triethylamine (100:1) as eluent to yield the corresponding secondary amine.

Confirmation of Identity of FT127 Product

FT127: yield 82 %; colourless oil

Calculated Atomic Composition: C 68.84, H 6.89, N 3.09; Measured Composition: C 69.16, H 7.00, N 3.00.

¹H NMR (CDCl₃): 1.33-1.49 (6 H, m), 1.75 (2 H, dt, *J* = 16 and 7 Hz), 2.58 (2 H, t, *J* = 7 Hz), 3.02 (2 H, t, *J* = 7 Hz), 3.29 (2 H, t, *J* = 7 Hz), 3.93 (2 H, t, *J* = 7 Hz), 6.85-7.06 (7 H, m), 7.26-7.32 (2 H, m), 7.55-7.69 (3 H, m), 7.91-7.94 (2 H, m).

Mass spectrometry of the compound using Electron Impact Ionisation to ionise the compound gave ions with the following mass to charge ratios: 453 (M⁺, 3 %), 312, 298, 285, 268, 198 (100 %), 186, 168, 141, 125, 100, 77.

Confirmation of Identity of FT129 Product

FT129: yield 78 %; colourless oil

Calculated Atomic Composition: C 63.30, H 6.91, N 3.69; Measured Composition: C 63.50, H 6.96, N 3.65.

^1H NMR (CDCl_3): 1.33-1.49 (6 H, m), 1.73 (2 H, dt, $J = 16$ and 7 Hz), 2.57 (2 H, t, $J = 7$ Hz), 3.02 (2 H, t, $J = 7$ Hz), 3.29 (2 H, t, $J = 7$ Hz), 3.90 (2 H, t, $J = 7$ Hz), 6.80-6.85 (2 H, m), 6.92-7.00 (2 H, m), 7.55-7.69 (3 H, m), 7.91-7.94 (2 H, m).

^{13}C NMR (CDCl_3): 25.92, 26.96, 29.21, 29.87, 43.16, 49.46, 56.16, 68.56, 115.48, 115.61, 115.92, 128.01, 129.40, 133.83, 139.61, 155.33, 158.83.

Mass spectrometry of the compound using Electron Impact Ionisation to ionise the compound gave ions with the following mass to charge ratios: 380 $[\text{M}+1]^+$ (1 %), 378 $[\text{M}-1]^+$ (1 %), 287, 268, 198 (100 %), 141, 125, 126, 112.

Confirmation of Identity of FT131 Product

FT131: yield 93 %; colourless oil

Calculated Atomic Composition: C 67.83, H 8.02, N 3.60; Measured Composition: C 67.89, H 8.06, N 3.55.

^1H NMR (CDCl_3): 1.31-1.49 (6 H, m), 1.75 (2 H, dt, $J = 16$ and 7 Hz), 2.18 (3 H, s), 2.22 (3 H, s), 2.56 (2 H, t, $J = 7$ Hz), 3.01 (2 H, t, $J = 7$ Hz), 3.29 (2 H, t, $J = 7$ Hz), 3.91 (2 H, t, $J = 7$ Hz), 6.62 (1 H, dd, $J = 9$ and 2.5 Hz), 6.70 (1 d, $J = 2.5$ Hz), 7.01 (1 H, d, $J = 9$ Hz), 7.55-7.69 (3 H, m), 7.90-7.94 (2 H, m).

^{13}C NMR (CDCl_3): 18.72, 19.97, 25.97, 26.98, 29.29, 29.89, 43.17, 49.48, 56.17, 67.87, 111.52, 116.27, 128.02, 128.48, 129.41, 130.32, 133.83, 137, 61, 139.61, 157.35.

Mass spectrometry of the compound using Electron Impact Ionisation to ionise the compound gave ions with the following mass to charge ratios: 389 (M^+ , 2 %), 268, 198 (100 %), 141, 126, 122, 107.

Confirmation of Identity of FT133 Product

FT133: yield 85 %, colourless oil

Calculated Atomic Composition: C 70.04, H 7.10, N 3.40; Measured Composition: C 69.80, H 7.13, N 3.38.

^1H NMR (CDCl_3): 1.35-1.62 (6 H, m), 1.92 (2 H, dt, $J = 16$ and 7 Hz), 2.56 (2 H, t, $J = 7$ Hz), 3.02 (2 H, t, $J = 7$ Hz), 3.28 (2 H, t, $J = 7$ Hz), 4.13 (2 H, t, $J = 7$ Hz), 6.89 (1 H, dd, $J = 8$ and 1.3 Hz), 7.33-7.68 (7 H, m), 7.78 (1 H, m), 7.90-7.94 (2 H, m), 8.27 (1 H, m).

^{13}C NMR (CDCl_3): 26.18, 27.00, 29.23, 29.92, 43.18, 49.48, 56.17, 68.02, 104.67, 120.07, 122.11, 125.11, 125.97, 126.37, 127.49, 128.02, 129.39, 133.81, 134.62, 139.61, 154.96.

Mass spectrometry of the compound using Electron Impact Ionisation to ionise the compound gave ions with the following mass to charge ratios: 411 (M^+ , 5 %), 268, 198 (100 %), 144, 126, 125, 115, 100.

Synthesis of FT134, FT135, FT136, FT137 - General Procedure

A solution of the corresponding secondary amine FT127, FT129, FT131 or FT133 (0.12 mmol) in dry dichloromethane (2 ml) was treated with triethylamine (0.25 ml) and finally at 0°C with trifluoroacetic anhydride (0.05 ml, 0.37 mmol). The reaction mixture was stirred at this temperature for 20 min and treated with methanol (0.25 ml). The solvents were evaporated under reduced pressure and the residue was dissolved in dichloromethane and washed with water. The organic extract was dried with sodium sulphate, evaporated to dryness and purified by flash chromatography on silica gel with *n*-hexane/ethyl acetate (2:1) to furnish the corresponding trifluoroacetamides FT134, FT135, FT136, and FT137.

Confirmation of Identity of FT134 Product

FT134: yield 93 %, amorphous solid.

^1H NMR (CDCl_3): 1.34-1.84 (8 H, m), 3.30-3.48 (4 H, m), 3.76 (2 H, m), 3.95 (2 H, m), 6.84-7.10 (7 H, m), 7.27-7.33 (2 H, m), 7.55-7.73 (3 H, m), 7.90-7.95 (2 H, m).

Mass spectrometry of the compound using Chemical Ionisation with Ammonia in the source to ionise the compound gave ions with the following mass to charge ratios: 567 $[M+NH_4]^+$ (100 %), 549 $[M]^+$, 475, 427, 399, 381, 186.

Confirmation of Identity of FT135 Product

FT135: yield 82 %; amorphous solid.

1H NMR ($CDCl_3$): 1.34-1.82 (8 H, m), 3.30-3.48 (4 H, m), 3.76 (2 H, m), 3.90 (2 H, m), 6.79-6.85 (2 H, m), 6.92-7.00 (2 H, m), 7.55-7.73 (3 H, m), 7.90-7.95 (2 H, m).

MS (CI, NH_3): 493 $[M+NH_4]^+$ (100 %), 475 $[M]^+$, 381, 353, 351, 325, 186.

Confirmation of Identity of FT136 Product

FT136: yield 98 %; amorphous solid.

1H NMR ($CDCl_3$): 1.30-1.81 (8 H, m), 2.19 (3 H, s), 2.23 (3 H, s), 3.30-3.48 (4 H, m), 3.77 (2 H, m), 3.92 (2 H, m), 6.64 (1 H, dd, $J = 9.5$ and 2.5 Hz), 6.71 (1 H, d, $J = 2.5$ Hz), 7.02 (1 H, d, $J = 9.5$ Hz), 7.55-7.73 (3 H, m), 7.90-7.95 (2 H, m).

Mass spectrometry of the compound using Chemical Ionisation with Ammonia in the source to ionise the compound gave ions with the following mass to charge ratios: 503 $[M+NH_4]^+$, 485 $[M]^+$, 383, 363, 335, 318, 241, 186, 122, 43 (100 %).

Confirmation of Identity of FT137 Product

FT137: yield 80 %; amorphous solid.

1H NMR ($CDCl_3$): 1.34-1.49 (2 H, m), 1.55-1.72 (4 H, m), 1.87-1.99 (2 H, m), 3.30-3.48 (4 H, m), 3.72 (2 H, m), 4.15 (2 H, m), 6.80 (1 H, dd, $J = 8.5$ and 1.5 Hz), 7.33-7.70 (7 H, m), 7.80 (1 H, m), 7.89-7.94 (2 H, m), 8.27 (1 H, m).

Mass spectrometry of the compound using Chemical Ionisation with Ammonia in the source to ionise the compound gave ions with the following mass to charge ratios: 525 $[(M+NH_4)]^+$, 100 %, $[508 (M+1)]^+$, 385, 383, 357, 340, 186, 160, 140.

Synthesis of FT142, FT143, FT146, FT147 - General Procedure

A solution of the corresponding secondary amine FT127, FT129, FT131 or FT133 (0.06 mmol) in dry dichloromethane (2 ml) was treated with triethylamine (0.1 ml) and finally at 0°C with methanesulphonyl chloride (0.1 ml, 0.13 mmol). The reaction mixture was stirred at this temperature for 20 min and treated with methanol (0.1 ml). The reaction mixture was diluted with dichloromethane and washed with water. The organic extract was dried with sodium sulphate, evaporated to dryness and purified by flash chromatography on silica gel with *n*-hexane/ethyl acetate (2:3) to furnish the corresponding sulphonamides FT142, FT143, FT146, and FT147.

Confirmation of Identity of FT142 Product

FT142: yield 86 %, amorphous solid.

¹H NMR (CDCl₃) 1.34-1.83 (8 H, m), 2.83 (3 H, s), 3.20 (2 H, t, *J* = 9 Hz), 3.41-3.47 (2 H, m), 3.57-3.62 (2 H, m), 3.94 (2 H, t, *J* = 7 Hz), 6.84-7.07 (7 H, m), 7.26-7.33 (2 H, m), 7.57-7.70 (3 H, m), 7.91-7.95 (2 H, m).

MS (DCI, NH₃): 549 [M+NH₄]⁺ 100 %, 531 M⁺, 409, 381, 223, 186.

Confirmation of Identity of FT143 Product

FT143: yield 88 %; amorphous solid.

¹H NMR (CDCl₃) 1.30-1.65 (6 H, m), 1.71-1.81 (2 H, m), 2.83 (3 H, s), 3.19 (2 H, t, *J* = 9 Hz), 3.41-3.47 (2 H, m), 3.56-3.62 (2 H, m), 3.91 (2 H, t, *J* = 7 Hz), 6.79-7.01 (4 H, m), 7.57-7.72 (3 H, m), 7.91-7.95 (2 H, m).

Mass spectrometry of the compound using Chemical Ionisation to ionise the compound gave ions with the following mass to charge ratios: 475 [M+NH₄]⁺, 458 [M+H]⁺, 363, 307, 238, 186.

Confirmation of Identity of FT146 Product

FT146: yield 89 %; amorphous solid.

^1H NMR (CDCl_3) 1.32-1.63 (6 H, m), 1.70-1.80 (2 H, m), 2.19 (3 H, s), 2.23 (3 H, s), 2.82 (3 H, s), 3.18 (2 H, t, $J = 9$ Hz), 3.41-3.47 (2 H, m), 3.55-3.62 (2 H, m), 3.92 (2 H, t, $J = 7$ Hz), 6.63 (1 H, dd, $J = 9.5$ and 3 Hz), 6.71 (1 H, d, $J = 3$ Hz), 7.02 (1 H, d, $J = 9.5$ Hz), 7.56-7.72 (3 H, m), 7.91-7.95 (2 H, m).

MS (DCI, NH_3): 485 $[\text{M}+\text{NH}_4]^+$ (100 %), 467 $[\text{M}]^+$, 363, 345, 317, 248, 186, 122.

Confirmation of Identity of FT147 Product

FT147: yield 99 %; amorphous solid.

^1H NMR (CDCl_3) 1.35-1.47 (2 H, m), 1.54-1.67 (4 H, m), 1.88-1.98 (2 H, m), 2.81 (3 H, s), 3.20 (2 H, t, $J = 9$ Hz), 3.41-3.47 (2 H, m), 3.55-3.62 (2 H, m), 4.14 (2 H, t, $J = 7$ Hz), 6.80 (1 H, d, $J = 8.5$ Hz), 7.32-7.51 (4 H, m), 7.54-7.7 (3 H, m), 7.80 (1 H, m), 7.91-7.95 (2 H, d, $J = 8$ Hz), 8.27 (1 H, m).

MS (DCI, NH_3): 507 $[\text{M}+\text{NH}_4]^+$, 490 $[\text{M}+1]^+$, 365, 339, 322, 206, 186 (100 %).

ESI-MS Analysis of FT 134, 135, 136, 137 and FT 142, 143, 146, 147

The eight compounds FT 134, 135, 136, 137 and FT 142, 143, 146, 147 were analysed on a Platform-LC quadrupole instrument (Micromass Ltd, UK) with an electrospray ionisation source. Solutions of each the markers were made up in a 50:50 mixture of water and acetonitrile. Ammonia was also present in the solvent at a concentration 0.2 %. Figures 11 to 18 show the negative ion mass spectra of FT 134, 135, 136, 137 and FT 142, 143, 146, 147 respectively. In each case there is no detectable molecular ion and a dominant peak in the spectrum corresponding to the negative ion cleavage product is identified in each case. In all of these spectra there are a number of additional peaks. The most significant of these occur at masses corresponding to the molecular mass of the uncleaved marker plus 45 daltons and plus 59 daltons which correspond to adducts of formate and acetate with the uncharged, uncleaved mass marker respectively. These were contaminants in the ion source of the mass spectrometer from previous use.

Addition of the amino groups of the protein albumin to the vinyl double bond of phenyl-vinylsulphone

In a mixture of an organic solvent such as acetonitrile and water, the solubility of albumin is increased by the addition of neutral salts. 7 mg of the protein were dissolved in 1 ml of a solvent mixture Acetonitrile/Water (75 % Acetonitrile (ACN)). To the cloudy solution were added 2 drops of NaCl 0.1 molar solution and 2 drops of triethylamine (TEA). The solution became clear after addition of the salt. To the clear solution of albumin was added 6 mg of phenyl-vinylsulphone (PVS) in 1 ml of ACN. The reaction mixture was stirred at room temperature overnight. The solvents were evaporated under reduced pressure. The solid residue was then dissolved in a small amount of methanol and the very small amount of salt that precipitated out was removed by filtration. The filtrate was reduced by evaporation and the final product was crystallised from ether to give 6.5 mg of a white solid. The solid was washed several times with distilled ethyl acetate to remove any excess PVS. The last washing was retained for analysis and the solid was dried under reduced pressure.

The GC-MS instrument was first calibrated and adjusted to perform the detection of the 125 m/z ratio peak that is characteristic for the ArSO^+ fragment from the parent molecular ion 168 m/z of PVS ($\text{CH}=\text{CH}_2\text{SO}_2\text{Ar}$). Neither blank solvent (pure ethyl acetate) nor the ethyl acetate from the last washing of PVS labelled albumin showed a 125 m/z peak. This means that the modified protein is not contaminated with any traces of free PVS.

Cleavage of the phenyl-vinylsulphone from the modified Protein

5 mg of the adduct (albumin-phenyl-vinylsulphone) in 2 ml MeOH was reacted with two drops of dimethyl sulphate for 2 h at room temperature. 5.2 mg of the quaternary ammonium salt dissolved in 2 ml dichloromethane (DCM) was treated with two drops of diisopropylethylamine (DIEA). The reaction mixture was stirred at room temperature for 2 h. The solvents then were evaporated and the residue was taken into Ethyl acetate, washed with water and dried over Na_2SO_4 . After evaporation of the solvent, the residue was dissolved in 1 ml of ethyl acetate and was analysed by GC-MS. A characteristic peak of m/z 125 for the

phenyl-vinylsulphone fragment was easily detected. The concentration of the cleaved compound was monitored and compared with an internal standard present in a known quantity; 2.5×10^{-6} mol of PVS was observed to be present, which is close to the amount of PVS that was linked to the protein during the modification.

Addition of Cysteine residues in Albumin and Bovine serum albumin to PVS

Albumin or Bovine serum albumin (Sigma) was dissolved in a mixture of acetonitrile/water. Triethylamine was added to the solution and left under nitrogen for 5 minutes. PVS was then added and the reaction mixture was stirred for only 15 min at room temperature. The PVS-labelled proteins gave a purple product when a portion was reacted with ninhydrin, indicating that free amines were present. After the labelling reaction, the solvent was evaporated at room temperature, and the residue from each protein was precipitated from ether and was recovered by filtration. The solid was washed several times with ethyl acetate and was dried under reduced pressure.

Thermal elimination of PVS from the Thio-labelled Albumin and BSA

Both samples (PVS thio-labelled albumin and BSA), when injected into the GC-MS as a solution (water/acetonitrile, 75 % ACN) gave distinctive peaks at m/z 125 corresponding to thermally eliminated sulphone. Experiments with PVS labelled albumin and BSA that had been separated by gel electrophoresis, with sodium dodecyl sulphate on a polyacrylamide gel (SDS-PAGE) and recovered using standard methods known in the art, also gave peaks at m/z 125 characteristic of PVS when analysed by GC-MS.

Treatment of thio-sulphones with aqueous and methanolic sodium hydroxide

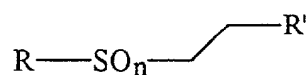
PVS labelled proteins are stable under the conditions of SDS-PAGE. It is desirable to be able to analyse proteins by iso-electric focusing, where proteins are separated on an electrophoretically maintained pH gradient. Proteins are separated according to their net charge and focus at the point where the pH leaves the protein with no net charge. Certain proteins are very basic and will focus at a very high pH and may be exposed to basic conditions for several hours. In order to assess whether PVS thiol derivatives are stable under

basic conditions, various PVS thio-labelled compounds were incubated for prolonged periods in the presence of 1 M aqueous NaOH and a methanolic solution of 1 M NaOH.

S-(phenyl-ethylsulphonyl)octylthiol in acetonitrile was treated with 1 M aqueous NaOH for 24 h with full recovery of the starting material after that time. When S-(phenyl-ethylsulphonyl)cysteine methyl-ester in acetonitrile was treated with 1 M aqueous NaOH for 24 h, slight cleavage of the sulphone was observed by UV stimulated emission from a Thin Layer Chromatography (TLC) plate after 2 h. But after 24 h, only 9.5 % of the sulphone had cleaved. The rest of the starting material was recovered as shown by the $^1\text{H-NMR}$. S-(phenyl-ethylsulphonyl)acetylcysteine methyl ester was synthesised by acetylation of S-(Phenyl-ethylsulphonyl)cysteine methyl-ester with acetic anhydride. This compound in acetonitrile was also treated with 1 M aqueous for 24 h. The very slight cleavage of the sulphone has been observed by TLC. But after 24 h, the percentage of the sulphone that was cleaved is only 7 %. The rest of the starting material was recovered and the $^1\text{H-NMR}$ spectrum was recorded. Similar results were obtained from analysis of these compounds in methanolic NaOH. It seems that a free amine in close proximity to the labelled thiol increases cleavage of the PVS-thiol derivative. Since the majority of thiol residues will not be at the N-terminus of a protein, this effect should not be a problem when using sulphones as labelling reagents for proteins. Moreover, in a typical iso-electric focusing separation, the most basic pH is about 12 whereas 1 M NaOH represents a pH of 14.

CLAIMS:

1. A method for characterising an analyte, which method comprises:
 - (a) providing a compound in which the analyte is attached by a cleavable linker to a reporter group relatable to the analyte, the compound having the following formula:

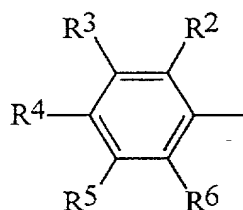


wherein either R comprises the reporter group and R' comprises the analyte, or R comprises the analyte and R' comprises the reporter group, and wherein n is 1 or 2;

- (b) cleaving the reporter group from the analyte; and
 - (c) identifying the reporter group, thereby characterising the analyte.
2. A method according to claim 1, wherein R and/or R' comprise a covalent linkage attaching the analyte and/or reporter group to the cleavable linker.
3. A method according to claim 2, wherein the covalent linkage is independently selected from a -CO-NH- group, an -NH-CO-NH- group, an -NH-CS-NH- group, a -CH₂-NH- group, an -SO₂-NH- group, an -NH-CH₂-CH₂- group, or an -OP(=O)(O)O- group.
4. A method according to any preceding claim, wherein R comprises, between the SO_n group and the reporter group or analyte, a substituted or unsubstituted aromatic cyclic group, aliphatic cyclic group, or heterocyclic group.
5. A method according to claim 4, wherein R comprises, between the SO_n group and the reporter group or analyte, a substituted or unsubstituted group selected from phenyl, pyridyl,

pyranyl, naphthyl, anthracyl, pyrenyl, or fused ring derivatives or heteroaromatic analogues of the above.

6. A method according to claim 5, wherein the phenyl group is a group having the following formula:



wherein one of R²-R⁶ comprises the reporter group or analyte, and the remaining R²-R⁶ groups are independently selected from a hydrogen, and a substituent, such as a D, F, methyl, methoxy, hydroxy or amino group.

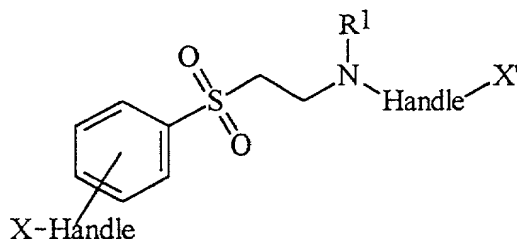
7. A method according to any preceding claim, wherein the R' group comprises a group selected from -S-, -SO-, -NR¹-, and -O- between the C atom that is in the β -position to the SO_n group, and the reporter group or analyte.

8. A method according to claim 7, wherein the R¹ group is an electron withdrawing group.

9. A method according to claim 8, wherein R¹ is a hydrogen atom, a halogen atom, or a substituent comprising a carbonyl group and/or a halogen atom.

10. A method according to claim 9, wherein R¹ is a fluorine atom, a chlorine atom, a bromine atom, an iodine atom, a trifluoroacetyl group, or a trifluoromethyl acetate group.

11. A method according to claim 1, wherein the compound has the following formula:



wherein R^1 is an electron withdrawing substituent, X comprises the reporter group and X' comprises the analyte, or X comprises the analyte and X' comprises the reporter group, and each Handle is the same or different, being either a single bond directly attaching the X groups to the phenyl ring and the N atom respectively, or a reactive group capable of attaching the X groups to the phenyl ring and the N atom respectively.

12. A method according to claim 12, wherein R^1 is selected from a hydrogen atom, a halogen atom, or a substituent comprising a carbonyl group and/or a halogen atom.

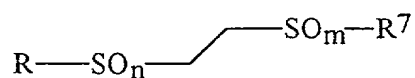
13. A method according to claim 12 or claim 13, wherein each Handle is independently selected from a -CO-NH- group, an -NH-CO-NH- group, an -NH-CS-NH- group, a -CH₂-NH- group, an -SO₂-NH- group, an -NH-CH₂-CH₂- group, or an -OP(=O)(O)O- group.

14. A method according to any preceding claim, wherein the analyte comprises a biological molecule.

15. A method according to claim 14, wherein the biological molecule is selected from a protein, a polypeptide, an amino acid, a nucleic acid, a nucleic acid base, a pharmaceutical agent or drug, a carbohydrate, a lipid, a natural product and a synthetic compound from an encoded chemical library.

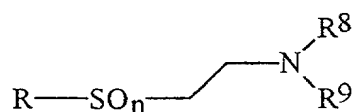
16. A compound according to claim 15, wherein the nucleotide, oligonucleotide or nucleic acid is natural, or is modified by modifying a base, sugar and/or backbone of the nucleotide, oligonucleotide or nucleic acid.

17. A method according to claim 15 or claim 16, wherein the analyte is an amino acid or a peptide comprising a cysteine group, and the compound is of the formula:



wherein m is 0 or 1 and the S atom attaching R^7 to the linker is the sulphur atom of the cysteine group, R^7 being the remainder of the amino acid or polypeptide.

18. A method according to claim 15 or claim 16, wherein the analyte is an amino acid or a peptide, and the compound is of the formula:



wherein the N atom is the nitrogen atom of an epsilon amino group of a lysine group, or is the nitrogen atom of an N-terminal alpha amino group, R^8 is selected from H, O or an N-protective group, R^9 being the remainder of the amino acid or polypeptide.

19. A method according to claim 15 or claim 16, wherein the analyte is an amino acid or a peptide comprising a serine, threonine and/or tyrosine group, and the compound is of the formula:



wherein the O atom is the oxygen atom from a hydroxyl group of the serine, threonine or tyrosine group, R¹⁰ being the remainder of the amino acid or polypeptide.

20. A method according to any preceding claim, wherein the reporter group comprises a mass marker detectable by mass spectrometry.

21. A method according to claim 20, wherein the mass marker comprises an oligoether or a polyether.

22. A method according to claim 21, wherein the oligoether or polyether is a substituted or unsubstituted oligo- or poly-arylether.

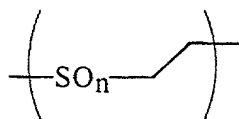
23. A method according to claim 21 or claim 22, wherein the oligoether or polyether comprises one or more fluorine atom or methyl group substituents, or one or more ²H or ¹³C isotopic substituents.

24. A method according to any of claims 20-23, wherein the mass marker comprises a metal ion-binding moiety.

25. A method according to claim 24, wherein the metal ion-binding moiety is a porphyrin, a crown ether, hexahistidine, or a multidentate ligand.

26. A method according to claim 25, wherein the metal ion-binding moiety is a bidentate ligand or is EDTA.

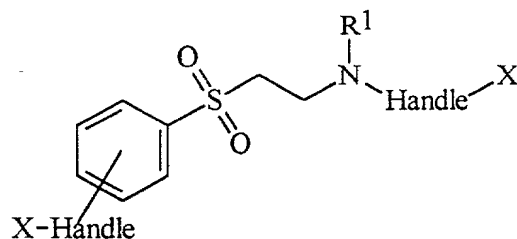
27. A method according to any of claims 24-26, wherein the metal ion-binding moiety is bound to a monovalent, divalent or trivalent metal ion.
28. A method according to claim 27, wherein the metal ion is a transition metal ion, or a metal ion of group IA, IIA or IIIA of the periodic table.
29. A method according to claim 28, wherein the metal ion is Ni^{2+} , Li^{+} , Na^{+} , K^{+} , Mg^{2+} , Ca^{2+} , Sr^{2+} , Ba^{2+} , or Al^{3+} .
30. A method according to any preceding claim, which method further comprises heating the linker to cleave off the reporter group.
31. A method according to any preceding claim, wherein the reporter group is a mass marker and the method further comprises cleaving off the mass marker in the mass spectrometer.
32. Use of a linker group in the characterisation of an analyte, to attach a reporter group to the analyte, wherein the linker group is cleavable and has the following formula:



wherein n is 1 or 2.

33. Use according to claim 32, wherein the analyte is as defined in any of claims 14-19.
34. Use according to claim 32 or claim 33, wherein the reporter group is as defined in any of claims 20-29.

35. A compound having the following formula:



wherein R^1 is an electron withdrawing substituent, X comprises the reporter group and X' comprises the analyte, or X comprises the analyte and X' comprises the reporter group, and each Handle is the same or different, being either a single bond directly attaching the X groups to the phenyl ring and the N atom respectively, or a reactive group capable of attaching the X groups to the phenyl ring and the N atom respectively.

36. A compound according to claim 35, wherein R^1 is selected from a hydrogen atom, a halogen atom, or a substituent comprising a carbonyl group and/or a halogen atom.

37. A compound according to claim 35 or claim 36, wherein each Handle is independently selected from a -CO-NH- group, an -NH-CO-NH- group, an -NH-CS-NH- group, a -CH₂-NH- group, an -SO₂-NH- group, an -NH-CH₂-CH₂- group, or an -OP(=O)(O)O- group.

38. A compound according to any of claims 35-37, wherein the analyte is as defined in any of claims 14-19.

39. A compound according to any of claims 35-38, wherein the reporter group is as defined in any of claims 20-29.

Fig.1.

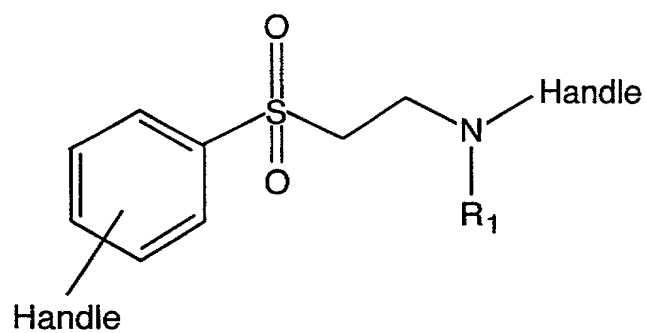


Fig.2.

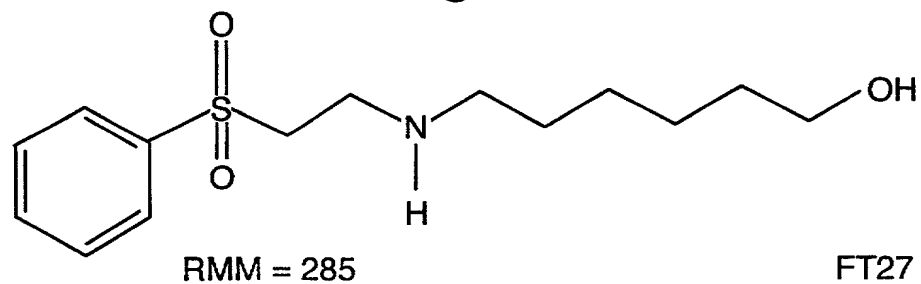
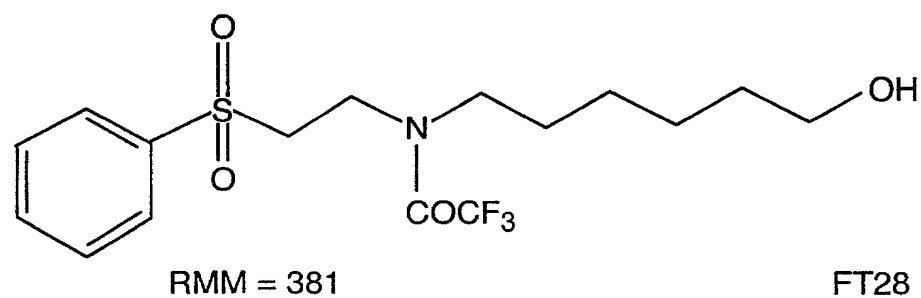


Fig.3.



2/16

Fig.4.

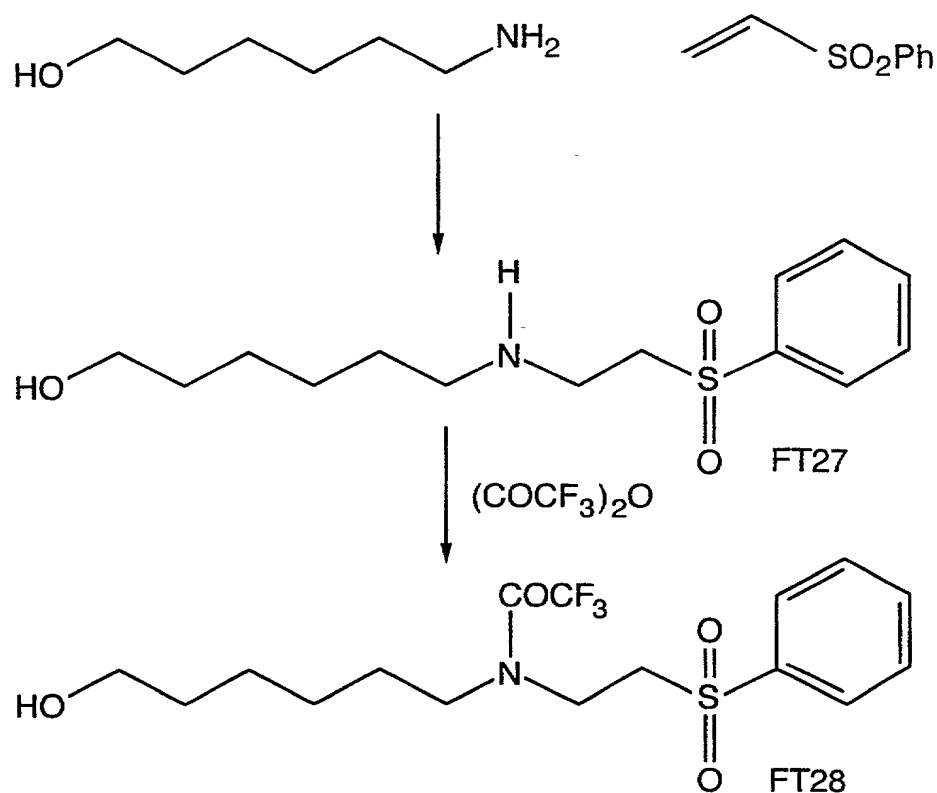
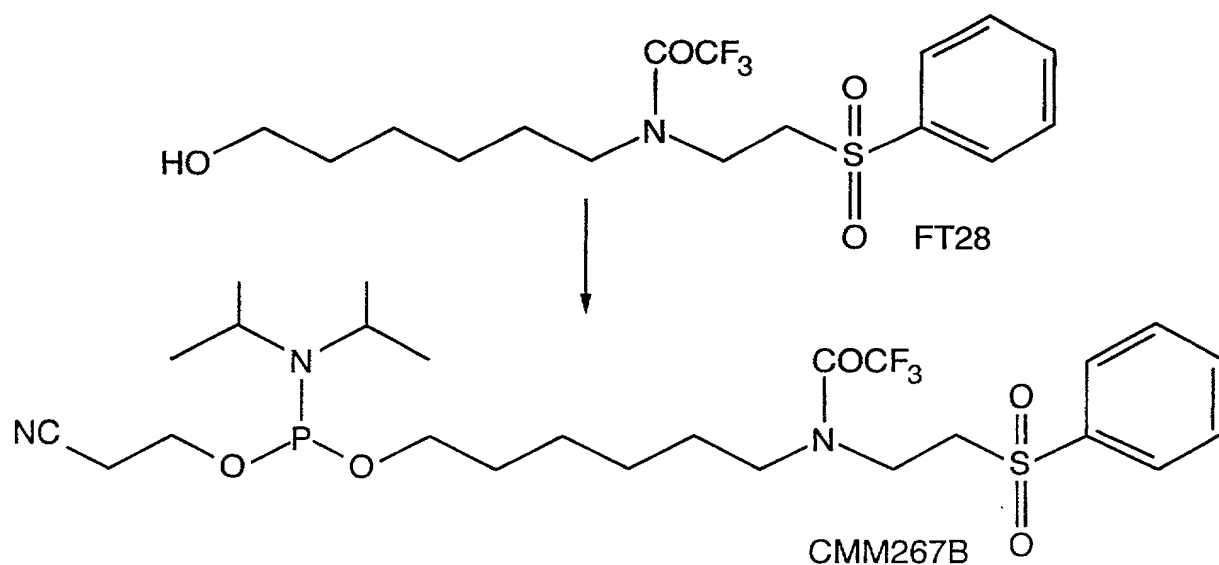
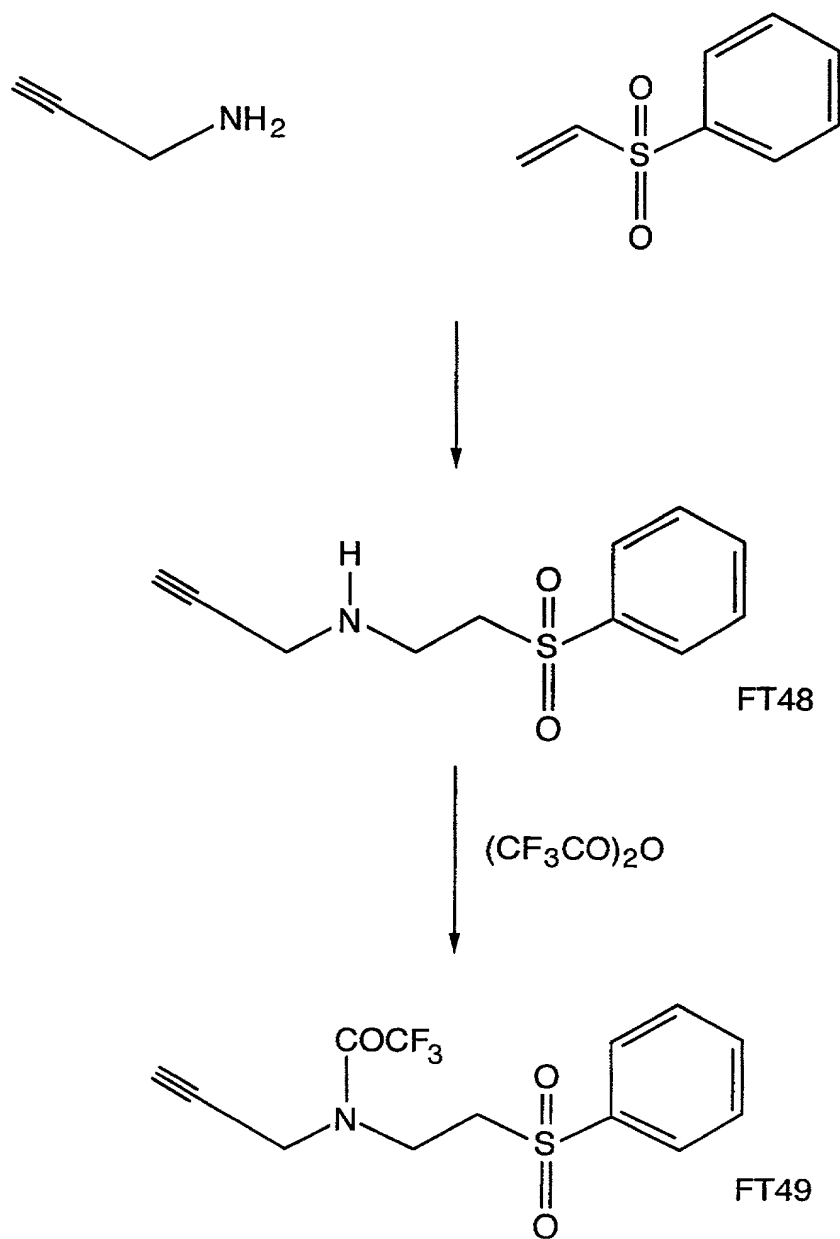


Fig.5.



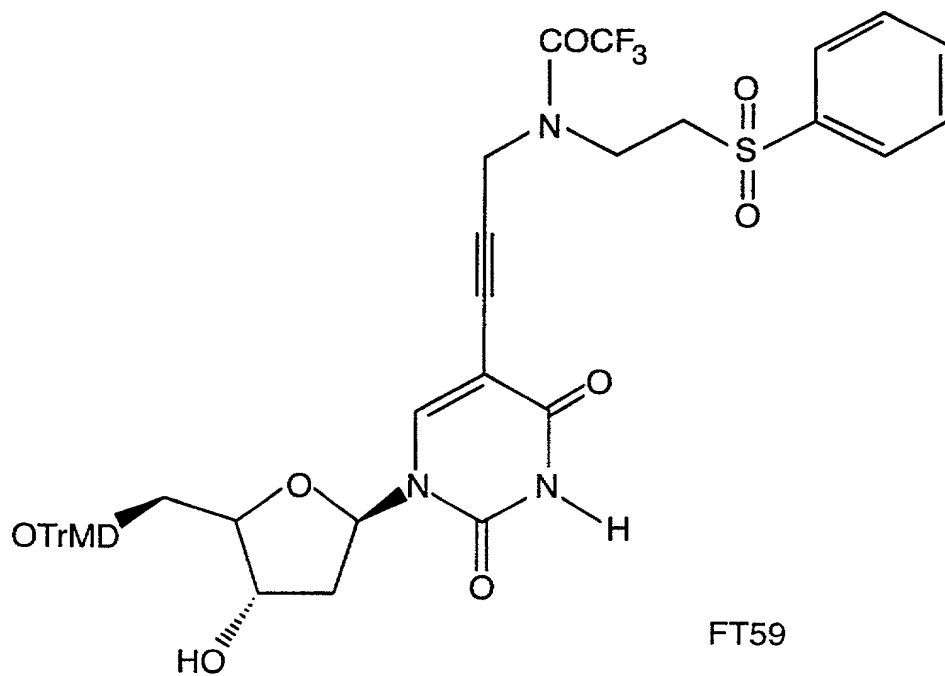
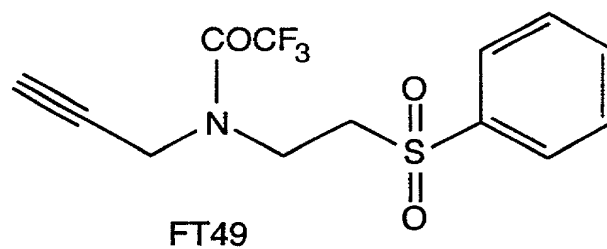
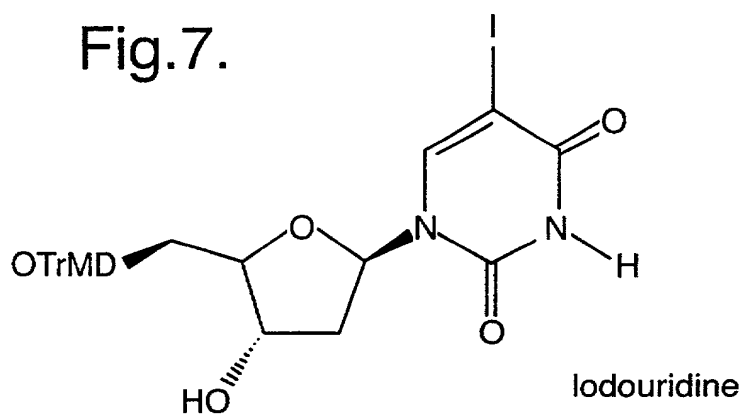
3/16

Fig.6.



4/16

Fig.7.



5/16

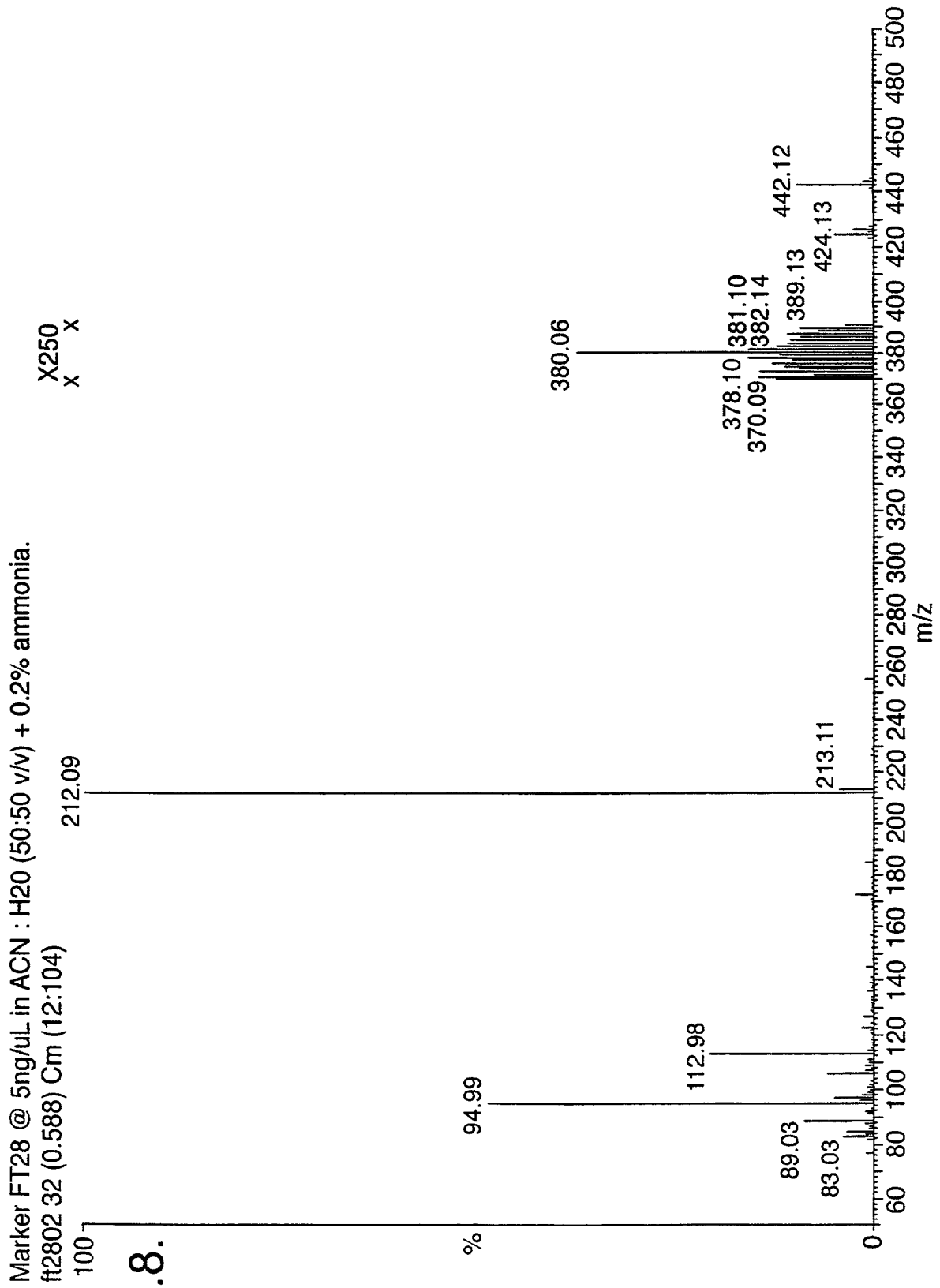
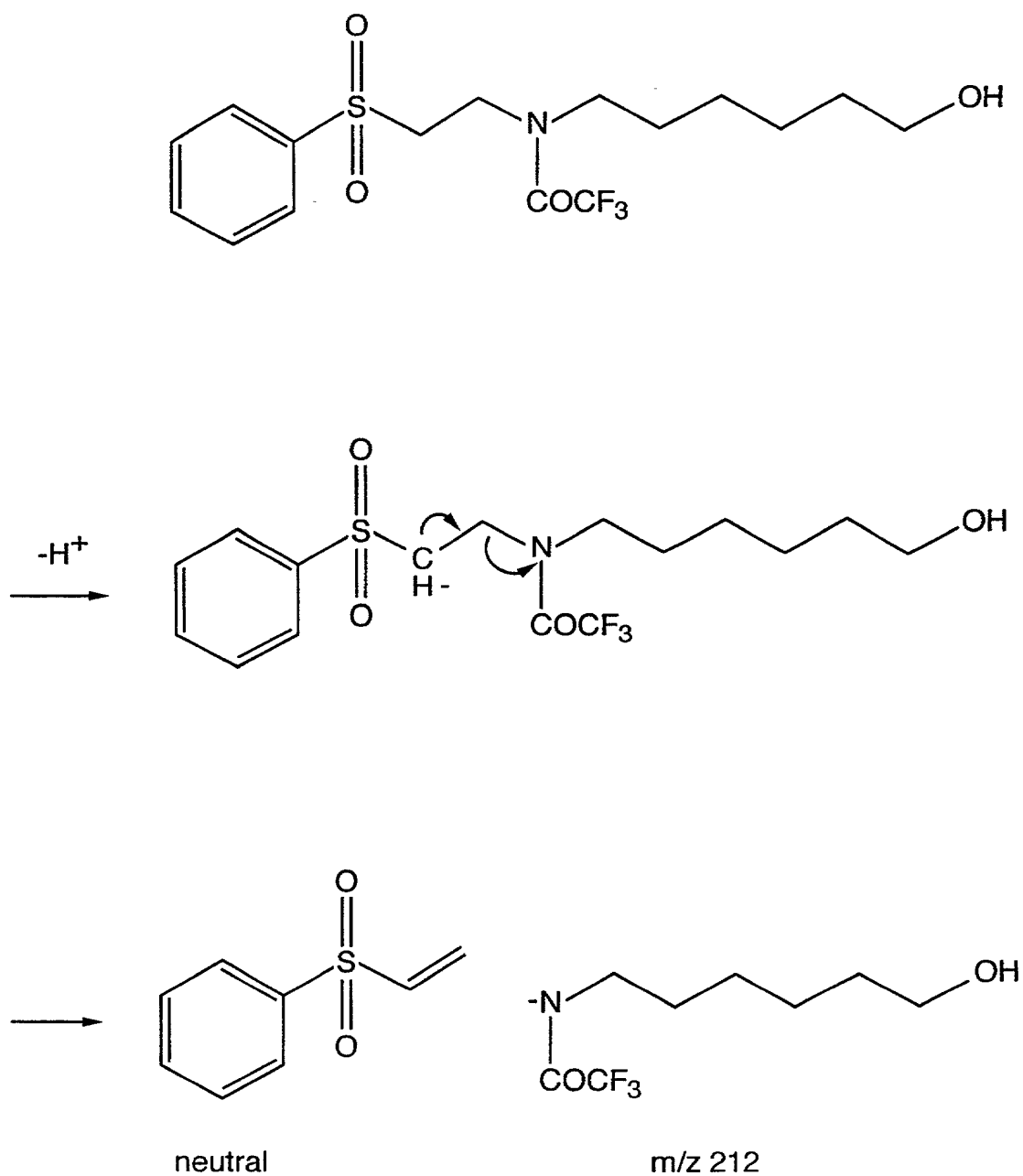


Fig.8.

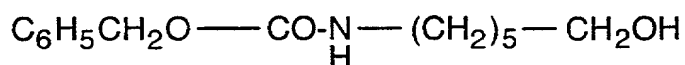
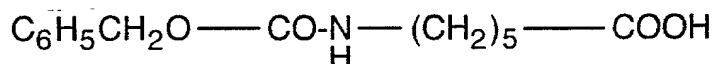
6/16

Fig.9.

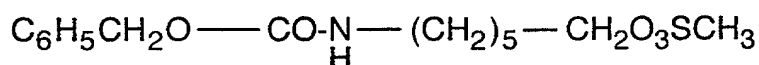


7/16

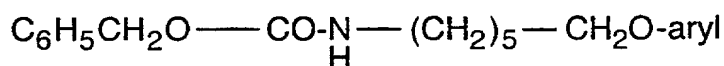
Fig.10.



FT116



FT117/2

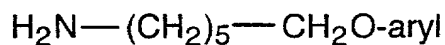


FT120: aryl = 4-phenoxyphenyl

FT121: aryl = 4-fluorophenyl

FT122: aryl = 3,4-dimethylphenyl

FT123: aryl = 1-naphthyl



FT126: aryl = 4-phenoxyphenyl

FT128: aryl = 4-fluorophenyl

FT130: aryl = 3,4-dimethylphenyl

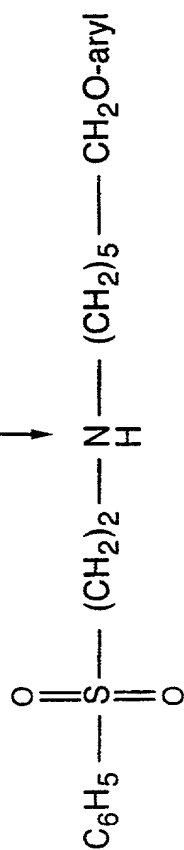
FT131: aryl = 1-naphthyl



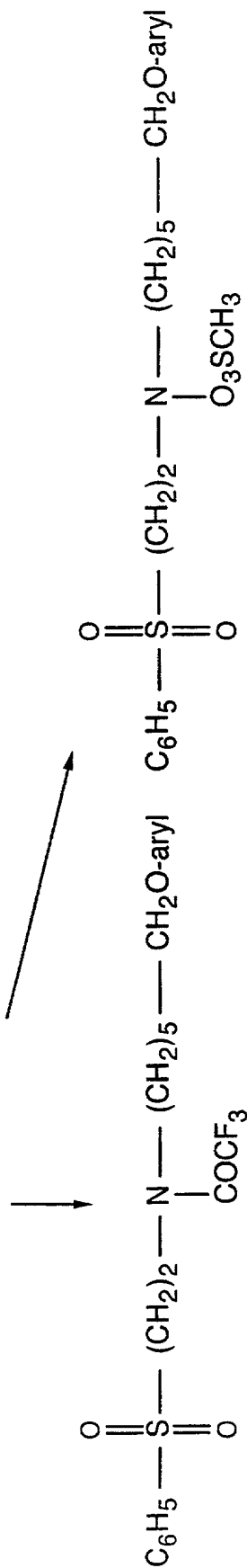
To Fig.10.(Cont)

Fig.10.(Cont)

From Fig.10.



FT127: aryl = 4-phenoxyphenyl
 FT129: aryl = 4-fluorophenyl
 FT131: aryl = 3,4-dimethylphenyl
 FT133: aryl = 1-naphthyl

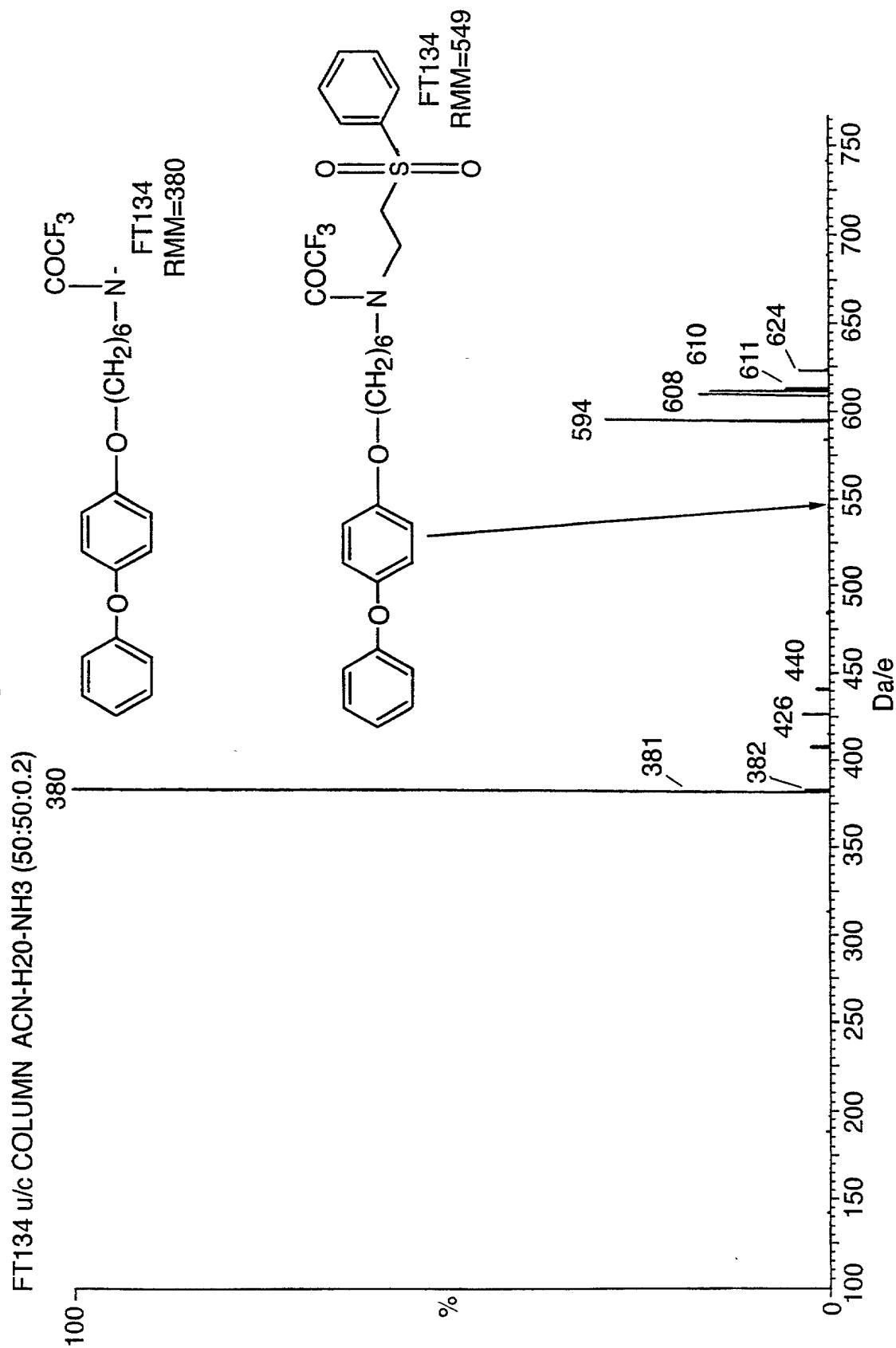


FT134: aryl = 4-phenoxyphenyl
 FT135: aryl = 4-fluorophenyl
 FT136: aryl = 3,4-dimethylphenyl
 FT137: aryl = 1-naphthyl

FT142: aryl = 4-phenoxyphenyl
 FT143: aryl = 4-fluorophenyl
 FT146: aryl = 3,4-dimethylphenyl
 FT147: aryl = 1-naphthyl

9/16

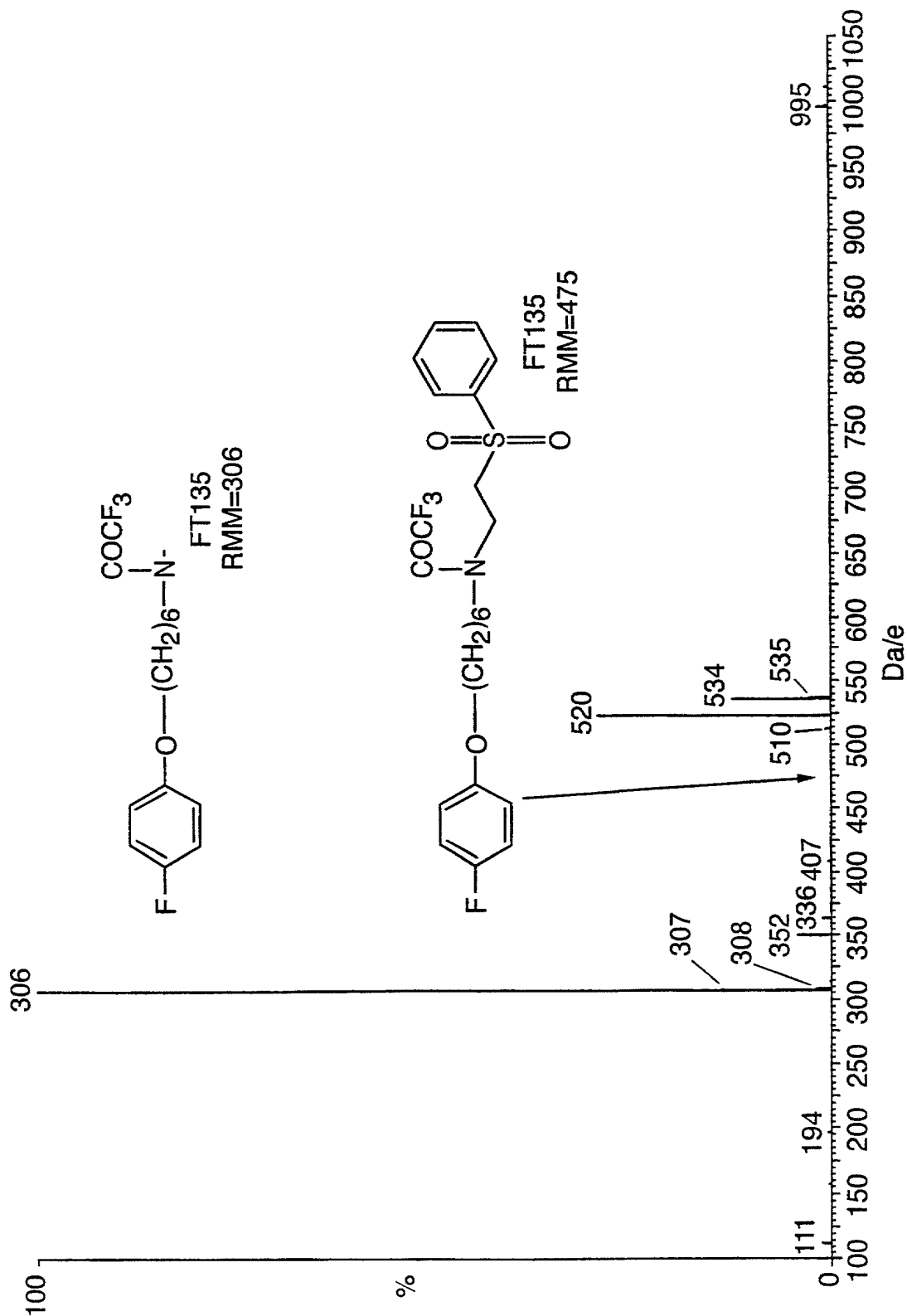
Fig.11.



10/16

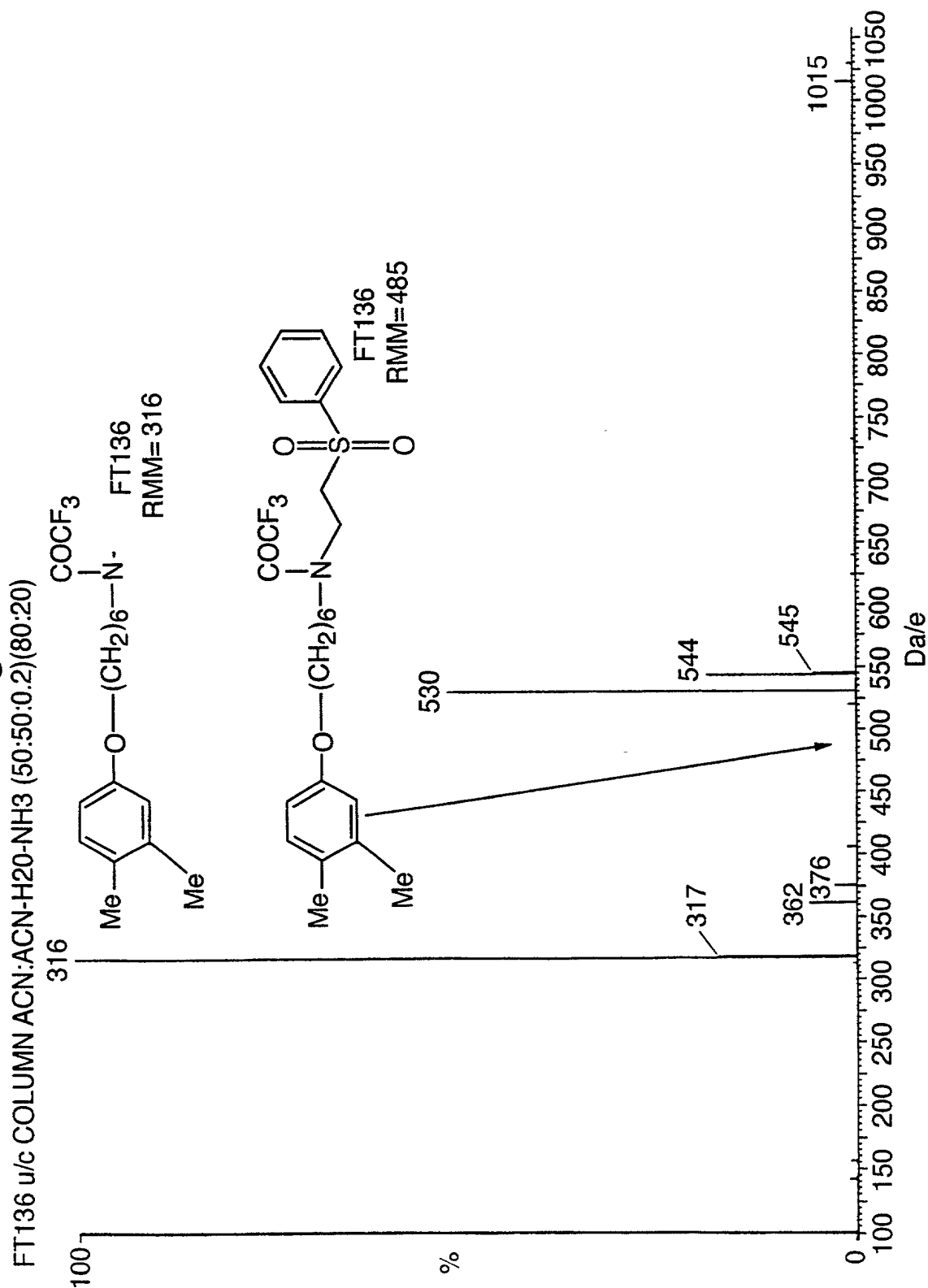
Fig.12.

FT135 u/c COLUMN ACN:H2O-NH3 (50:50:0.2)(80:20)



11/16

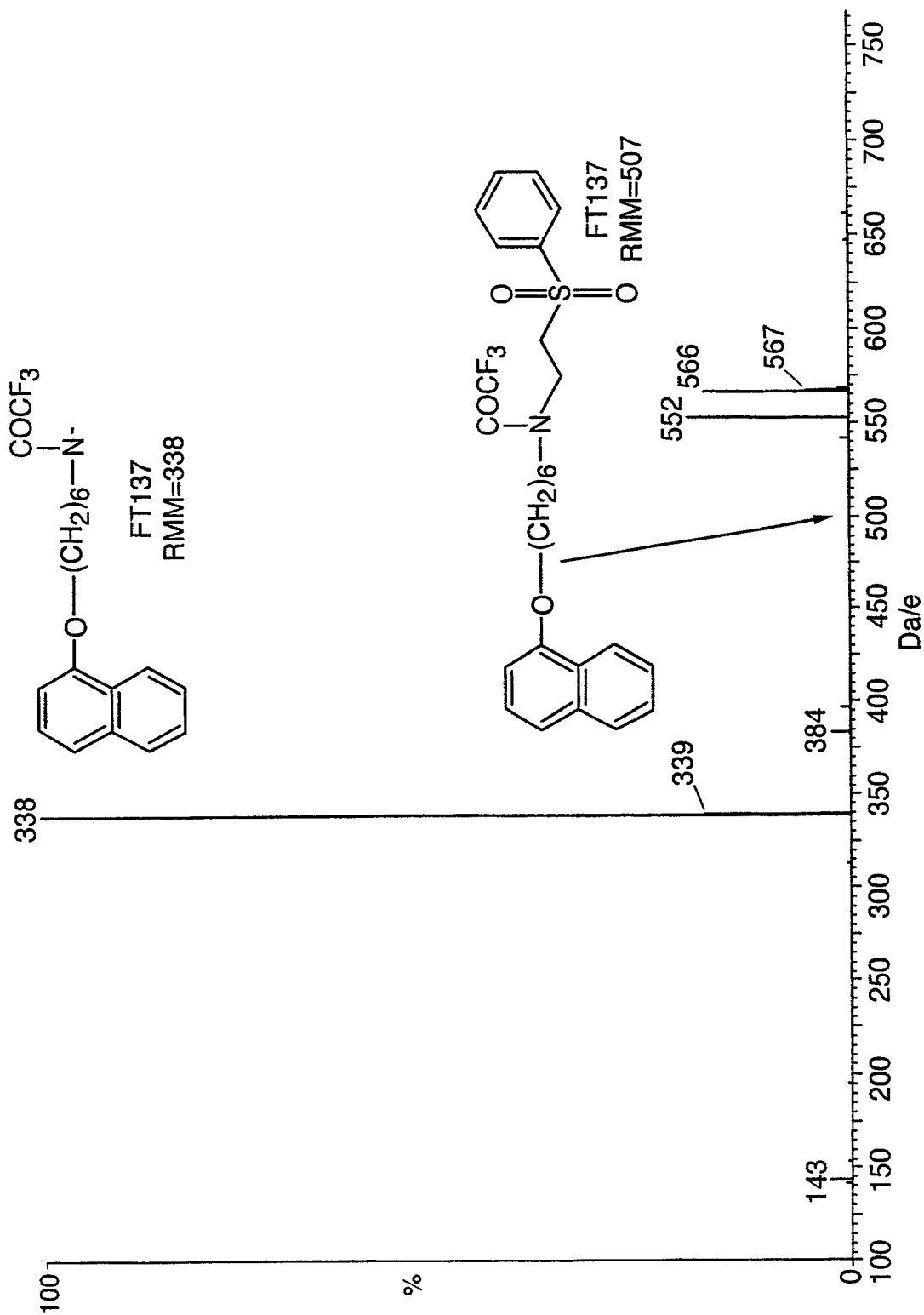
Fig.13.



12/16

Fig.14.

FT137 u/c COLUMN ACN:ACN-H2O-NH3 (50:50:0.2)(80:20)



13/16

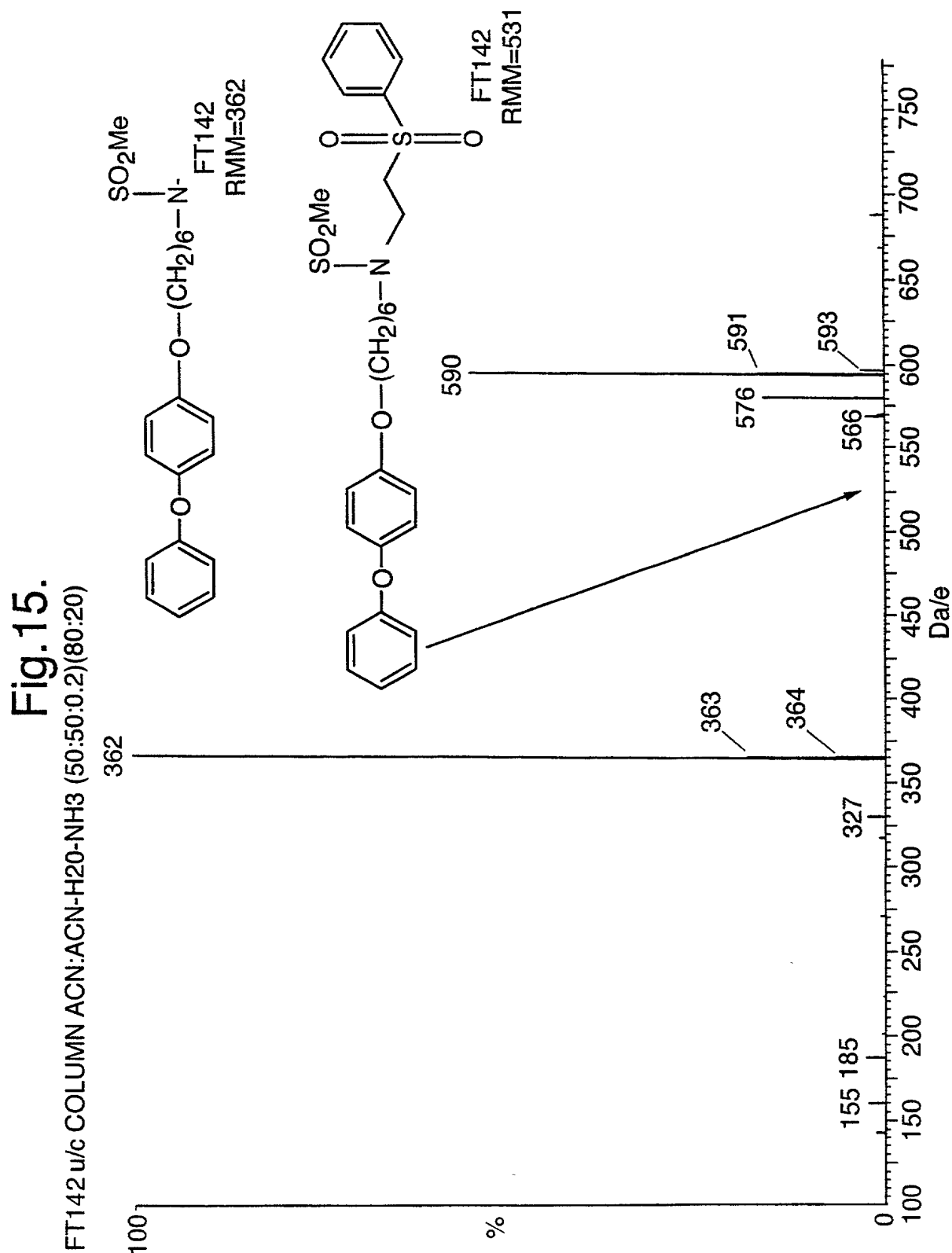


Fig.16.

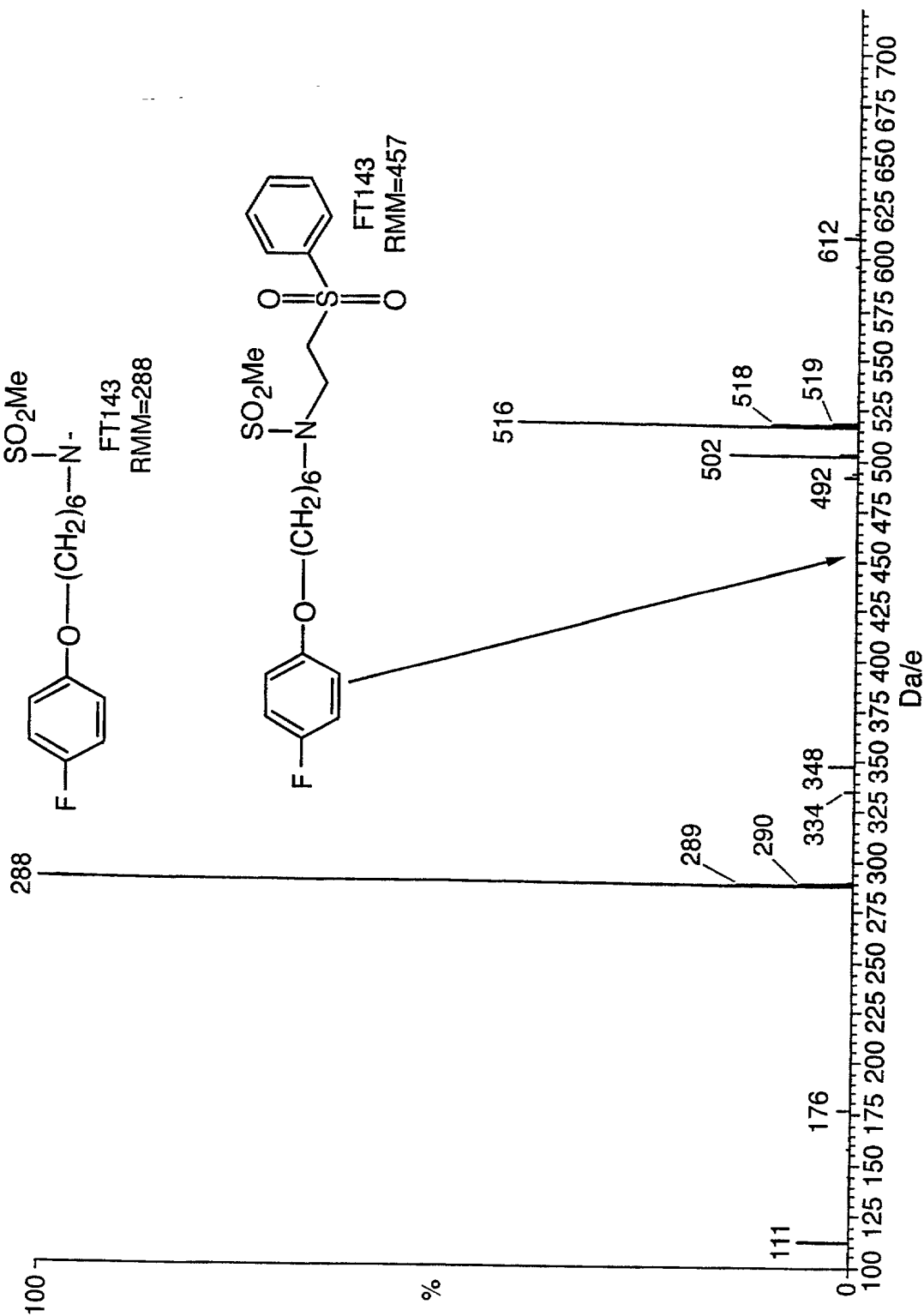
FT143 u/c COLUMN ACN: ACN-H₂O-NH₃ (50:50:0.2) (80:20)

Fig.17.

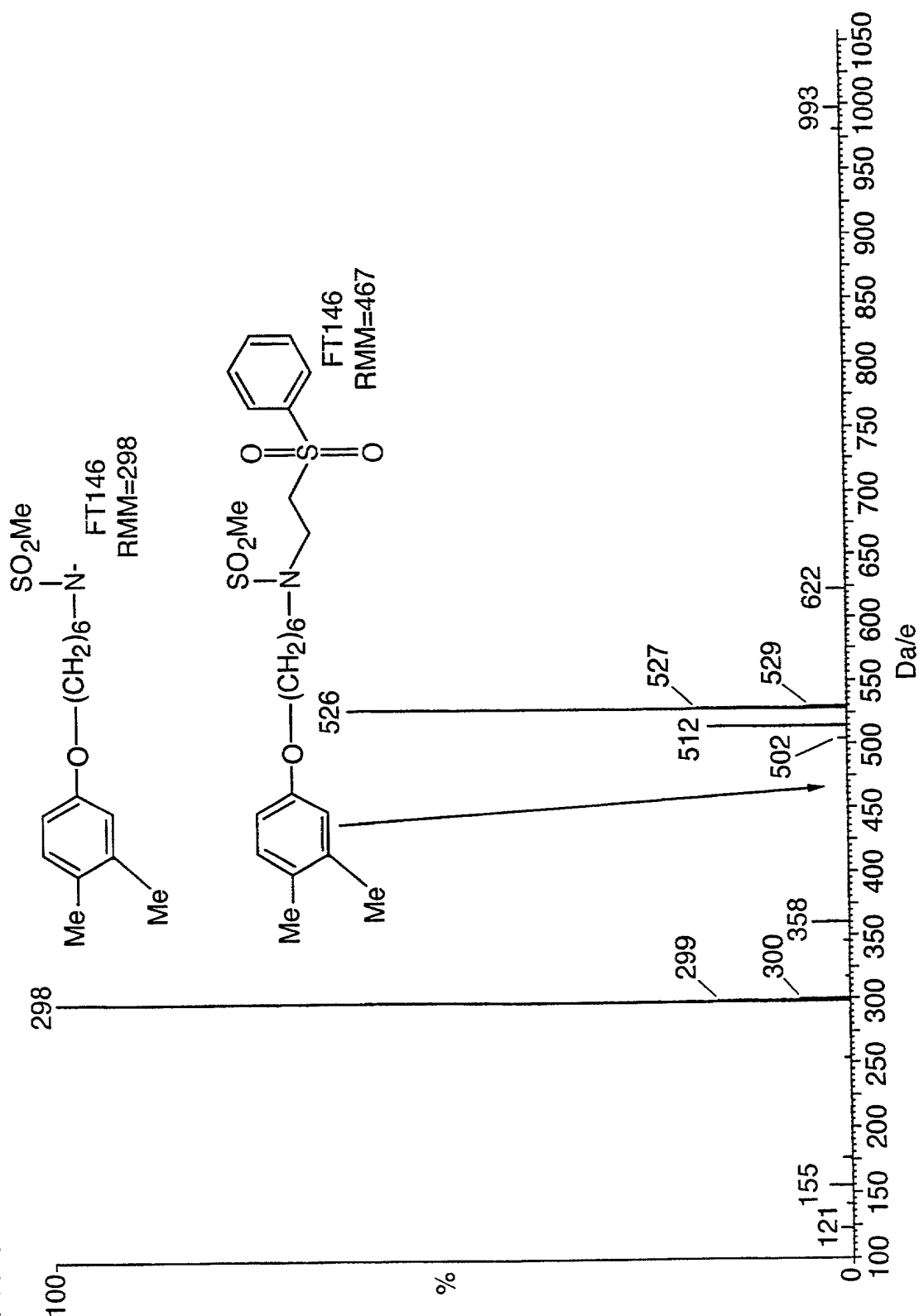
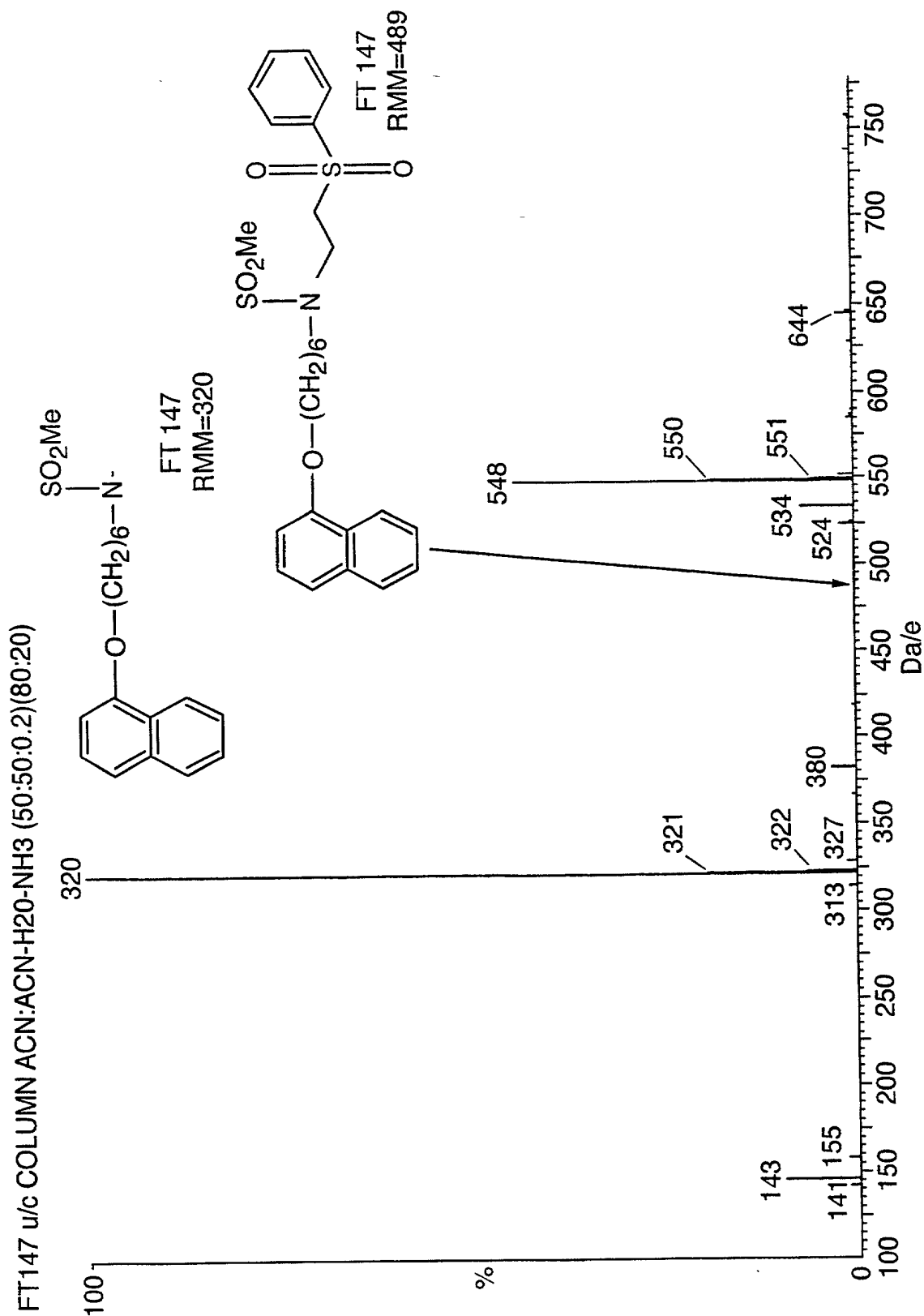
FT146 u/c COLUMN ACN:H₂O-NH₃ (50:50:0.2)(80:20)

Fig.18.



FOR UTILITY/DESIGN
CIP/PCT NATIONAL/PLANT
ORIGINAL/SUBSTITUTE/SUPPLEMENTAL
DECLARATIONS

RULE 63 (37 C.F.R. 1.63)
DECLARATION AND POWER OF ATTORNEY
FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PW
FORM

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the INVENTION ENTITLED ARYLSULFONE LINKERS FOR MASS SPECTROMETRIC ANALYSIS

the specification of which (CHECK applicable BOX(ES))

X A. ☒ is attached hereto.
BOX(ES) → B. ☒ was filed on January 16, 2001 as U.S. Application No. /
→ C. ☐ was filed as PCT International Application No. PCT/ / on

and (if applicable to U.S. or PCT application) was amended on

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. Except as noted below, I hereby claim foreign priority benefits under 35 U.S.C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International Application which designated at least one other country than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International Application, filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application:

PRIOR FOREIGN APPLICATION(S)

Number	Country	Day/MONTH/Year Filed	Date first Laid-open or Published	Date Patented or Granted	Priority NOT Claimed
PCT/GB99/02257	International	13 July 1999	20 January 2000		
9815163.2	Great Britain	13 July 1998			

If more prior foreign applications, X box at bottom and continue on attached page.

Except as noted below, I hereby claim domestic priority benefit under 35 U.S.C. 119(e) or 120 and/or 365(c) of the indicated United States applications listed below and PCT international applications listed above or below and, if this is a continuation-in-part (CIP) application, insofar as the subject matter disclosed and claimed in this application is in addition to that disclosed in such prior applications, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which became available between the filing date of each such prior application and the national or PCT international filing date of this application:

PRIOR U.S. PROVISIONAL, NONPROVISIONAL AND/OR PCT APPLICATION(S)

Application No. (series code/serial no.)	Day/MONTH/Year Filed	Status	Priority NOT Claimed
		pending, abandoned, patented	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Pillsbury Winthrop LLP, Intellectual Property Group, 1100 New York Avenue, N.W., Ninth Floor, East Tower, Washington, D.C. 20005-3918, telephone number (202) 861-3000 (to whom all communications are to be directed), and the below-named persons (of the same address) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to delete names/numbers below of persons no longer with their firm and to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct the above firm and/or a below attorney in writing to the contrary

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G. Lloyd Knight	17698	G. Paul Edgell	24238	Michael R. Dzwonczyk	36787	Robert J. Walters	40862
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George M. Sirilla	18221	Timothy J. Klima	34852	Jack S. Barufka	37087		
Donald J. Bird	25323	David A. Jakopin	32995	Adam R. Hess	41835		
Dale S. Lazar	28872	Mark G. Paulson	30793	William P. Atkins	38821		
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Glenn J. Perry	28458	Richard H. Zaitlen	27248	Robin L. Teskin	35030		

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Date: 16 Feb 2001

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"X" box ☒ FOR ADDITIONAL INVENTORS, and proceed on the attached page to list each additional inventor.

☐ See additional foreign priorities on attached page (incorporated herein by reference).

Atty. Dkt. No. P0276611

(M#)

FILED PCT/PTO

16 MAR 2001

Completion Under R.

09/743746

9. (No.) Certified copy (copies): ☐ attached; ☐ previously filed (date) _____
in U.S. Application No. _____ / filed on _____
10. Small Entity Status ☐ is Not claimed ☒ is claimed (file PAT-256 if this is the first claim of Small Entity Status)
11. ☐ Attached:
12. ☐ Preliminary Amendment:

THE FOLLOWING FILING FEE IS BASED ON CLAIMS AS FILED LESS ANY ABOVE CANCELLED

				Large/Small Entity		Fee Code
13. Basic Filing Fee Design Application				\$320/\$160		106/26
Not Design Application				\$710/\$355	+0	101/201
14. Total Effective Claims		minus 20 =		x \$18/\$9	+0	103/203
15. Independent Claims		minus 3 =		x \$80/\$40	+0	102/202
16. If <u>any proper</u> multiple dependent claim (ignore improper) is present, (Leave this line blank if this is a reissue application)				\$270/\$135	+0	104/204
17. Surcharge for filing Declaration/filing fee late				\$130/\$65	+65	105/205
18. FILING FEE ENCLOSED =				\$65		
19. Original due date: March 21, 2001						
20. Petition is hereby made to extend the original due date to (1 mo)				\$110/\$55 =	+0	115/215
Cover the date this response is filed for which the requisite fee (2mos)				\$390/\$195 =		116/216
is attached (3mos)				\$890/\$445 =		117/217
(4mos)				\$1390/\$695 =		118/218
21. If "non-English" box 3 is X'd, add Rule 17(k) processing fee				\$130	+0	139
22. If "assignment" box 5 is X'd, add recording fee.				\$40	+0	581
23. Petition Fee for				\$130	+0	
24. TOTAL FEE ENCLOSED =				\$65		

Our Deposit Account No. 03-3975
Our Order No. 068800

0276611

C#

M#

CHARGE STATEMENT: The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-18 (missing or insufficiencies only) now or hereafter relative to this application and the resulting Official document under Rule 20, or credit any overpayment, to our Account/Order Nos. shown in the heading hereof for which purpose a duplicate copy of this sheet is attached. This **CHARGE STATEMENT** does not authorize charge of the issue fee until/unless an issue fee transmittal form is filed.

03/20/2001 UEDUVIJE 00000016 09743746

01 FC:254

65.00 GP

**Pillsbury Winthrop LLP
Intellectual Property Group**

1100 New York Avenue, NW

By Atty: **Milan M. Vinnola for
Robin L. Teskin**

Reg. No. 45,979

Ninth Floor
Washington, DC 20005-3918
Tel: (202) 861-3000
Atty/Sec: RLT/kmh

Sig:

Milan M Vinnola

Fax: (202) 822-0944
Tel: (202) 861-3623

NOTE: File in duplicate with PTO receipt (PAT-103A) and attachments

DECLARATION AND POWER OF ATTORNEY

(continued)

ADDITIONAL INVENTORS:

(3) INVENTOR'S SIGNATURE:

Date: 19-02-01

Robert	Alexander W.	Johnstone
First	Middle Initial	Family Name
Residence	Bebington	Great Britain
City	State/Foreign Country	Country of Citizenship
Mailing Address	39 Poulton Road, Bebington, Great Britain	
(include Zip Code)	L63 9LD	

(4) INVENTOR'S SIGNATURE:

Date:

First	Middle Initial	Family Name
Residence		
City	State/Foreign Country	Country of Citizenship
Mailing Address		
(include Zip Code)		

(5) INVENTOR'S SIGNATURE:

Date:

First	Middle Initial	Family Name
Residence		
City	State/Foreign Country	Country of Citizenship
Mailing Address		
(include Zip Code)		

(6) INVENTOR'S SIGNATURE:

Date:

First	Middle Initial	Family Name
Residence		
City	State/Foreign Country	Country of Citizenship
Mailing Address		
(include Zip Code)		

(7) INVENTOR'S SIGNATURE:

Date:

First	Middle Initial	Family Name
Residence		
City	State/Foreign Country	Country of Citizenship
Mailing Address		
(include Zip Code)		

(8) INVENTOR'S SIGNATURE:

Date:

First	Middle Initial	Family Name
Residence		
City	State/Foreign Country	Country of Citizenship
Mailing Address		
(include Zip Code)		

(9) INVENTOR'S SIGNATURE:

Date:

First	Middle Initial	Family Name
Residence		
City	State/Foreign Country	Country of Citizenship
Mailing Address		
(include Zip Code)		